ABSTRACT

CONTROLLING THE DENSITY OF DISULFIDE CROSS-LINKS IN KERATIN BIOMATERIALS FOR BONE REGENERATION: BIOACTIVITY AND MECHANISM AND DURATION OF BMP-2 DELIVERY

by Salma Haque

Effective methods for the treatment of bone injuries that do not heal (non-union) or heal slowly (delayed union) after reduction is a clinical challenge. The use of a collagen sponge to deliver recombinant human bone morphogenetic protein 2 (rhBMP-2) has been shown to be an effective substitute to autografts and allografts for the regeneration of new bone tissue. However, the FDA has limited its approved use to lumbar spinal fusion, open tibial fracture, and craniomaxillofacial injuries, presumably due to ectopic bone growth and other side effects that result from supraphysiological doses of rhBMP-2. Based on our previous findings in femur and mandibular defects, we explored keratin as an alternative carrier system to the existing collagen-based rhBMP-2 delivery system. We have previously used a strategy in which we modulated the levels of disulfide crosslinking in keratin hydrogels based on the extracted form of keratin. This thesis seeks to understand how rhBMP-2 delivered from the keratin hydrogels reaches to cells to achieve bioactivity. *In vitro* studies were conducted to assess the bioactivity of the soluble rhBMP-2 and matrix-bound rhBMP-2. rhBMP- 2 that remains associated with the keratin carrier is more bioactive than rhBMP-2 that is released from the carrier as a soluble “drug”.

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ABBREVIATIONS

ALP- Alkaline phosphatase

ACS- Absorbable collagen sponge

ECM- Extracellular matrix

KOS- Keratose

KTN- Kerateine

rhBMP-2- Recombinant Human Bone Morphogenetic Protein-2

rhBMP-7- Recombinant Human Bone Morphogenetic Protein-7

TGFβ- Transforming growth factor-Beta

FGFs- Fibroblast Growth Factors

PDGF- Platelet-Derived Growth Factor

RGD - Arginine-Glycine-Aspartic Acid

LDV - Leucine-Aspartic Acid-Valine

IFPs - Intermediate Filament Proteins

IGF-1 - insulin-like growth factor 1

MSCs - Mesenchymal Stem Cells

DMB - Demineralized Bone Matrix

TERM - Tissue engineering/regenerative medicine

RFU – Relative Fluorescence Units

VEGF - Vascular endothelial growth factor

bFGF - Fibroblast Growth Factor

IAM - iodoacetamide

MKTN - modified kerateine

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CHAPTER I- Introduction: Bone Fracture, Bone Regeneration, and use of Keratin Biomaterials as a Carrier for rhBMP-2 in Promoting Bone Fracture

Salma Haque

I.1. Overview: Biomedical Need for Bone Regeneration Products and the Development of Synthetic Bone Graft Materials by Delivery of rhBMP-2

The human skeletal system has the innate capability of healing fractures and forming new bone tissue. There are two key factors in play during these processes: (1) bone resorption and (2) new bone deposition. Unfortunately, under conditions including malnutrition, aging and traumatic/high energy, cancer, and congential defects, the skeletal system may be unable to maintain homeostasis between bone resorption and bone deposition [1]. A number of disease states are associated with a net loss of bone tissue. These include osteomalacia, hyperparathyroidism, osteoporosis and corticosteroid-induced osteopenia. These conditions lead to increased risk of bone fracture. Among, the 6.5 million fractures that occur in United States each year, approximately 15% show delayed or non-union fracture healing [4]. The number of bone injuries expected in the year 2050 worldwide is estimated between 7.3 million and 21.3 million with the cost of annual treatment to lie between $153.5 billion and $446.3 billion [5]. As such, it can be expected that delayed or non-union will represent on the order of 1 million cases by this time.

Autografts and allografts have been the clinical paradigm for the treatment of delayed/non-union. More recently, much effort has been focused on the development of alternative (synthetic) bone graft substitutes, which may be considered part of the field of tissue engineering/regenerative medicine (TERM). TERM strategies seek to use a combination of materials (i.e., scaffolds), cells (i.e., implanted or recruited from those already existing in the patient), and signals (e.g., chemical cues). Already, these strategies have yielded sales of approximately 2.5 billion dollars per year [6]. Therefore, the development of new strategies that can achieve improved bone regeneration following injury would be advantageous from both a clinical treatment perspective and an economic perspective.

In terms of chemical cues to aid in TERM strategies for bone regeneration, many bone inducing molecules have been discovered. Bone morphogenetic protein (BMPs) are a class of compounds found to play key roles in bone homeostasis, and several members of this family have been found to be sufficient for promoting bone regeneration. After their initial discovery, a key step forward in the use of these molecules was the ability to generate them in a recombinant

fashion. Recombinant human bone morphogenetic protein 2 (rhBMP-2) (Infuse; Medrontic Sofamor Danek) and recombinant human bone morphogenetic protein 7 rhBMP-7 (OP-1; Stryker Biotech) are two commercial forms of BMP that have received FDA approval for several clinical uses [7]. rhBMP-2 is relevant for both clinical applications and also for the studies described in this thesis work, since it has been found to be sufficient and necessary for bone regeneration. BMP-2 plays an important role in stimulating the proliferation of bone forming cells and helps to induce differentiation in osteoblast leading to an increase in the production of bone matrix [8]. Infuse® Bone Graft (Medrontic) is currently approved for use in the treatment of spinal fusion, open tibial fracture, and some craniofacial applications. It uses the combination of a bovine collagen that is made from bovine Achilles tendon [9] with rhBMP-2 as its growth factor that leads to osteogenesis through the differentiation of the preosteoblasts (immature and undifferentiated bone cells) to osteoblasts (bone forming cells) [10]. When this collagen carrier is implanted at the defect sites, it is degraded within several days of implantation, leading to burst release of rhBMP-2. While these materials have promoted successful clinical outcomes, the rapid resorption of ACS and the rapid release of rhBMP-2 can lead to ectopic bone growth and numerous other side effects [11]. Thus, while this is an important product, it is possible that it has been used for limited applications because of these side-effects. Materials that can accomplish the same effects with fewer side effects may allow a more broad use of such TERM strategies for bone repair beyond spinal fusion, tibial fracture, and the craniofacial applications for which Infuse is approved.

Stated differently advances in the controlled release of rhBMP-2 or bone inducing molecules would be beneficial for the regeneration of bone tissue. In this thesis, we describe a part of the research of our group into the use of keratin as an alternate carrier to collagen. Keratins are a natural polymer that have been used for variety of biomedical applications including bone repair [1], hemostasis [2], burn healing [3], and nerve regeneration [4], [5] which can be fabricated into fibers, gels, films, sponges and scaffolds [12][13]. In this thesis, we describe our approach to make use of keratin proteins, characterized by a high content of cysteine amino acid residue, to tune the rate of the rhBMP-2 carrier degradation and assess the bioactivity of rhBMP-2 associated with keratin compared to collagen. We first provide additional background information related to bone formation and fracture repair processes**.**

I.2. Key Cells Involved in Bone Formation and Repair

Bone consists of metabolically active cells that makes it a dynamic biological tissue [14]. In most mammalian bone has four surfaces or bone envelopes: the periosteal, endocortical, trabecular and Haversian (or intracorical) envelopes [15]. Bone tissue contains osteogenetic precursor cells, osteoblasts, osteoclasts, osteocytes and the hematopoietic elements that make up the bone marrow. At all the nonresorptive bone surfaces, osteoprogenitor cells are present that contribute to the deepest layer of the periosteum and endosteum. The periosteum consists of a thick outer layer of connective tissue known as "fibrous layer", containing dense and irregular tissue. The thin inner layer of the periosteum, which is poorly defined, is termed as an "osteogenic layer", comprised largely of osteogenic cells. The endosteum on other hand, lacks fibrous tissue and contains only a single layer of osteogenic cells. The differentiation of these progenitor cells leads to the development of mature osteoblasts [8], [14]. Osteoblasts are active, bone-forming cells derived from mesenchymal stem cells that generate new bone tissue. Bone derives its strength and rigidity from osteoid, secreted by osteoblasts. During homeostasis, bone formation by osteoblasts is balanced by bone resorption from osteoclasts. [14], [15]. Mature osteoblasts that are trapped within the bone matrix are called osteocytes. Osteocytes regulate the conduction of mechanical and biochemical signals to other osteocytes or bone lining cells [15]. Osteoclasts are multinucleated, bone resorbing cells that differentiate from the hematopoietic stem cells in the bone marrow. They are controlled by hormonal and cellular mechanisms. For example, parathyroid hormone (PTH) is the hormone that is involved in increasing the number and activity of osteoclasts that leads to greater resorption of calcium and phosphate ions resulting in bone resorption [16]. These cells functions by producing hydrolytic enzyme that results in the formation of Howship lacunae- a shallow erosive pits on the surface of the bone [14]. Ultimately, the appropriate interplay between each of these cell types is critical to bone homeostasis, and bone repair strategies that achieve this homeostasis are most likely to achieve success.

I.3. Bone Structure

Bone is generally classified into one of three types: woven bone, cortical bone or cancellous bone. Woven bone is the bone that is formed during embryonic development, fracture

healing and in some disease conditions. It consists of collagen bundles with lining of osteoblasts in the vascular spaces [14]. Cortical bone constitutes the thick outer shell of the bone and is remodeled by means of vascular channels from the woven bone. It is also known as compact or lamellar bone. Osteons are the structural unit of lamellar bone that aids in the mechanical strength of the bone. Cancellous bone also known as trabecular or spongy bone is made up of network of spaces that contains hematopoietic element and branching struts called trabeculae. The function of the trabeculae is to provide structural support. Volkmann canals are connected adjacent to osteons [17]. Ultimately, any bone fracture repair product should recapitulate with as high a fidelity as possible, this native bone architecture. In order to achieve this with rhBMP-2 delivery, we are suggesting that the dosage and timing of delivery must ultimately be optimized.

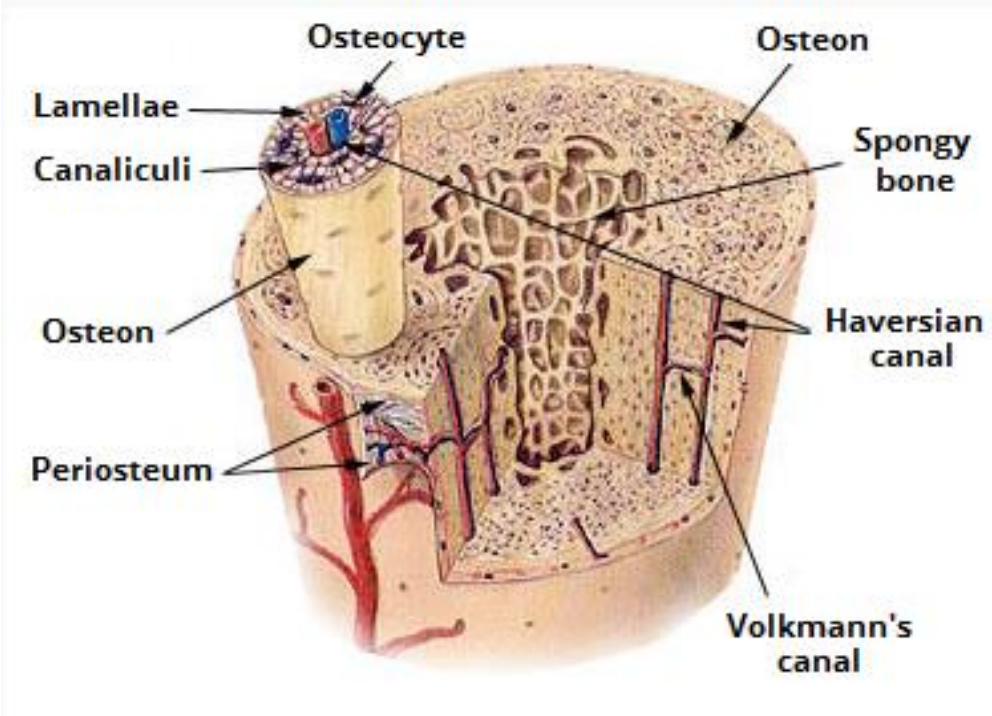

Figure 1: Structure of long bone. See text for additional information on the role of each component of the bone structure. Image used with permission [18].

I.4. Repair Process for Bone Fracture

Natural healing of bone is a complex process that requires the combination of biological and mechanical factors [19]. Normal function of the skeletal system involves breaking down of

old bone tissue and synthesizing new bone tissue to replace the resorbed tissue. Bone has innate ability to repair itself following injury [20] and remodeling of bone tissue is continuously occurring [14]. The process of bone fracture repair is generally described by three phases: (1) inflammatory, (2) reparative and (3) remodeling, as shown in Figure 2. In the initial phase of inflammation, hematoma is generated which contains both bone marrow cells as well as cells from peripheral and intramedullary blood [21]. This inflammatory response triggers hematoma to coagulate around the fracture ends and thereby leading to the formation of callus [22]. Debris which were created form the fracture itself are cleared up by phagocytic cells like polymorphs, histiocytes and mast cells that infiltrate the fracture site, thus forming a cavity which are later occupied by the osteoprogenitor cells.

In the reparative phase, a fibrin rich granulated tissue forms after the formation of hematoma. Here, the formation of callus tissue depends upon the recruitment of specific mesenchymal stem cells (MSCs) which proliferate and differentiate into osteogenic cells that then synthesize and deposit bone-specific matrix proteins which is composed of 90% type I collagen and 10% ground substance containing noncollagenous proteins, glycoproteins, proteoglycans, peptides, carbohydrates, and lipids. Strength and rigidity of the bone is obtained by the mineralization of osteoid [14]. MSCs that are used to fill the gap at the fracture site can be obtained from soft tissues, cortex, periosteum and bone marrow [23]. These cells are then replaced by other cells which helps in the formation of immature woven bone that unites the fracture site. After the synthesis of this newly mineralized bone and gaining mechanical strength and rigidity, fracture callus formation occurs. In case of endochondral ossification, cartilage may have formed near the peripherial region of the callus, which bridges the gap temporarily. This callus later forms the bone with the supply of blood vessels [24]. MSCs plays an important role by differentiating into chondroblasts thus giving rise to calcified cartilage matrix and subsequently to new bone and bone marrow. This is a complex cascade of events and if the balance of the process is not correct, proper bone healing may not occur. It is also noteworthy that the repair process involves other cell types such as chondrocytes for cartilage template deposition. These cells are also susceptible to BMP-2 stimulation, and it is therefore important to understand the bioactivity levels of BMP-2 when delivered to a fracture site as part of a TERM bone graft strategy.

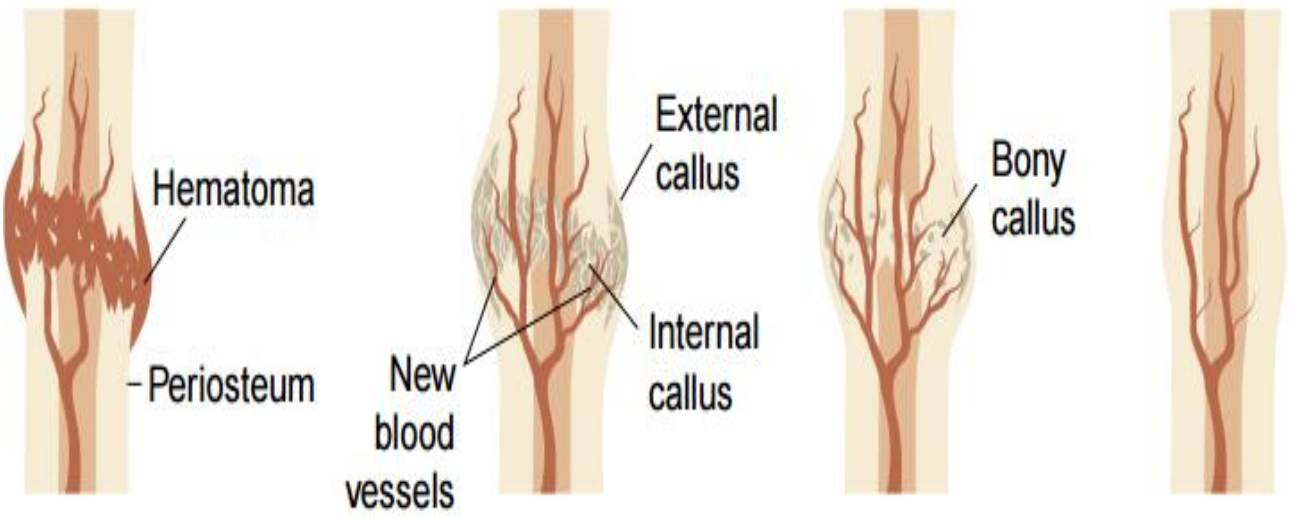

Figure 2: Phases of bone fracture repair: (A) Formation of hematoma in inflammation phase (B and C) Repair phase involving the formation of soft and hard callus formation (D) Remodeling of bone [6]. Used with permission.

I.5 Fracture Repair and Related Problems

As noted above, the human body must maintain the balance between the cells that lead to bone formation and bone resorption. The biological and mechanical properties are restored following normal healing of the bone [14]. Unfortunately, under certain conditions like malnutrition, aging, and traumatic injuries or defects resulting from cancer, trauma, congential diseases, the skeletal system sometimes fails to achieve this homeostasis [14], [20]. In cases of fracture when the body is unable to regenerate bone in order to heal the defect, a condition called non-union results. In this case, different surgical methods are required to attempt to achieve union (bridging) of the fracture. [20]. Grafts which are being used to treat injuries include autografts (i.e. the bone graft from the host itself), allografts (i.e. the graft from the same species), and xenografts (i.e. from different species) [1], as well as synthetic bone grafts.

To promote the healing of the fractures, autologous bone grafting is considered the clinical "gold standard" [19]. However, the use of autografts is subject to limited supply as these are generally obtained from iliac crest. In addition, the second site of injury (iliac crest) and donor site morbidity is a problem [1]. Failure rates of up to 30% have been described with autologous bone grafting due to resulting complications.

Likewise, allografts have potential problems including transmission of infectious disease and risk of graft rejection due to immunology response [1] as the graft involves the transplant of tissue from one individual to the other of the same species. The source of the grafts can either be living or cadaveric. These grafts undergoes processing methods like demineralization, freezing and freeze drying the grafts [6]. In order to reduce the bacterial and infectious risks associated with allografts, disinfection or sterilization techniques are required which can involve the mixtures of washing (with or without pressurization), centrifugation with different chemicals such as alcohol and detergent, and combining antibiotics. Low-dose radiation or gamma radiation are also used as the sterilizing technique [7].

Due to the problems associated with autografts and allografts, alternative repair strategies are needed, and approaches that use biomaterials are common. In developing these types of materials, it is considered ideal to have materials that are osteoconductive, osteoinductive and achieve osteogenesis. Osteoinduction is the ability to induce preosteoblast (i.e. undifferentiated and pluripotent cells) for osteogenesis. Osteogenesis is involved the formation of new bones. Osteoconduction provides surface for bone growth [26].

Alternative methods to grafts involves the use of bio-inert materials like gold, aluminum, tantalium silicone, stainless steel, titanium, elastomers, acrylic resins (methyl(methacrylate) and ceramics, each of which has been used to treat bone defects [25]. Bioinert materials generally lead to the formation of the fibrous tissue layer between the material and the bone during the final stage of the healing processes. However, these materials have proved to be ineffective due to lack of osteoinduction and limitations in the failure of the implant-tissue interface [27]. That is, these materials act as a bone substitute, but they do not harness the regenerative capacity of bone to fully heal and regenerate. Synthetic bone graft consisting of calcium phosphate ceramics have also been used to support in the healing of bone. These ceramic materials have

osteoinductive properties and do not have the risks of disease infection and are available in a so-called "off-the shelf" form. These materials are generally inexpensive but lead to very limited growth of tissue and can lead to fracture and wear [27], [28].

An alternative approach is to develop biomaterials that truly participate in or facilitate bone regeneration as part of a tissue engineered bone regeneration strategy. Tissue engineering is generally considered to consist of the triad of materials (scaffolds), cell signals (mechanical or chemical) and cells. In the case of bone, cells may be delivered exogenously or endogenous cells that are recruited to the implant site may be sufficient to achieve repair. These TERM strategies typically make use of chemical cues and have the capability to achieve regeneration of bone. Ultimately, bone regenerated by a tissue engineering approach should achieve an architecture which has similarity to uninjured bone (see above). This approach is advantageous as the material properties can be tailored for the better delivery of bone inducing molecules and cells and it also requires no secondary site for surgical methods. The properties of the biomaterial carrier for the chemical cues and the chemical cues themselves, however, are important design considerations.

I.6. Bone Morphogenetic Protein 2 as a Potent Osteoinductive Factor

Biological factors like transforming growth factor-Beta (TGFβ), bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), platelet-derived growth factor (PDGF), insulin-like growth factors (IGFs) and LIM mineralization protein-1 (LMP-1), have been examined for their roles in bone formation and thus as potential chemical cues to promote bone regeneration as part of a tissue engineering approach [7]. The most promising class of osteoinductive factors that play an essential role in native bone healing are the bone morphogenetic proteins which were first discovered by Marshall Urist in 1965 [8]. The BMPs belong to the transforming growth factor beta (TGFβ) superfamily of proteins. Not all the BMPs that have been discovered possess the significant osteogenic properties apart from BMP-2,4,6,7 and 9 [29]. In the 1970's/1980's these proteins were produced recombinantly because the amount of BMP that could be extracted from xenogeneic or allogeneic sources was too low to be clinically useful. Among all the different types of BMP been discovered, the recombinant human

forms of BMP-2 and BMP-7 have been used in clinical trials with potential of inducing bone *in vivo*.

BMP proteins are initially expressed in an inactive form and are activated by proteolytic cleavage by proteases releases the mature BMP proteins [30]. The activity of the BMPs and recombinant BMPs require the presence of disulfide-linked dimers [31]. The interaction of the two heterodimeric complex with the two transmembrane serine/threonine kinase complex leads to the signal transduction of BMP-2 [32]. BMP-2 direct the formation of tissue in body by initiating the mesenchymal stem cells to penetrate to the injury site, mostly leading to the differentiation process and then the induction of bone or cartilage [29], [33]. Recombinant human BMP-2 have a short half-life and are rapidly inactivated *in vivo* [34]. Also, there can be growth of the bone at undesired locations, which is called ectopic bone growth [29]. Therefore, there is a need for carrier vehicles that do not degrade rapidly and would allow sustained release and prevent the rapid diffusion of rhBMP-2 from the fracture site, thus increasing the efficacy [35]. This makes it necessary to design a carrier that would help in sustained release of rhBMP-2.

Only rhBMP-2 has the ability to induce osteoblastic differentiation of mesenchymal stem cells [7], but challenges associated with its use such as edema and ectopic bone formation have led to investigate of alternative methods to deliver rhBMP-2 to bone defect site. The topic of this thesis work is a comparative investigation of a keratin-based carrier system to an existing collagen-based carrier.

I.7. Existing Bone Morphogenetic Protein-2 Carrier Systems

An appropriate carrier system is required for the delivery of rhBMP-2 due to its short half-life and low *in vivo* bioactivity [36]. An ideal carrier would be the one that meets the following criteria: ability to retain rhBMP-2, ability to be replaced with the formation of bone tissue, mechanically stable, disease free and having low toxicity [14]. Various carrier systems have been reported including demineralized bone matrix (DBM) [37], poly[L-lactide-co-glycolide] coplolymer (PGLA) microparticles with blood clot (PMS/blood) [38], polylactic-co-glycolic acid, poly-glycolic acid, hydroxyapatite, tricalcium phosphate [39][40][41], ceramized coral [29], bovine bone or materials infused with bone marrow cells and collagen [42].

Currently, the U.S. Food and Drug Administration (FDA) approved carrier for rhBMP-2 is an absorbable formaldehyde-cross linked type I collagen sponge known by various tradenames including Infuse (Medtronic) [9]. During surgery rhBMP-2 in aqueous solution is adsorbed onto the collagen sponge [43]. The combination of rhBMP-2 and absorbable sponge collagen (ACS) has been approved by FDA for anterior-approach lumbar fusion affected by sinus augmentation, localized alveolar ridge augmentation, degenerative disease for the defects related with extraction sockets, and healing of acute open tibial shaft fractures [44]. The Infuse product as compared to autograft has shown improvement in healing with reduction in pain as well as eliminating the need for second site of injury, as described in several clinical studies [45], [46]. It believed that collagen degrades very rapidly *in vivo*, thus potentially losing the ability of the rhBMP-2 carrier to act as an osteoconductive matrix to promote bone regeneration. This rapid degradation also leads to a burst release and supra-physiological dosage of [10] rhBMP-2 leading to ectopic bone formation [47]. These can result in serious, life threatening medical complications, particularly when used for off-label applications such as those in the cervical spine [48]. Also, the adverse effects during pregnancy and lactation, fluid formation, edema [4], [42], bone resorption, and implant fractures has been reported [34]. Risk of ectopic bone formation is related to the features of carrier, dosage of rhBMP-2 and any barrier that would prevent the relocation of rhBMP to the injury site [43]. As noted above, the use of alternative carrier systems for rhBMP-2 could help to overcome some of these problems. Our group is investigating the use of keratin as a possible alternative carrier for rhBMP-2 [11].

I.8. Use of Hydrogels for the Controlled Release of Growth Factors

There is a clinical need for improved long-term performance of various therapeutics such as rhBMP-2 [50] not only for bone repair, but for TERM approaches to the repair of other tissues. Based upon the cell types and its internal/external physiological environment, the regenerative capability of tissue varies. When therapeutic drugs (which we define broadly here to include exogenously delivered growth factors, proteins, and small molecules) are delivered, challenges include the ability to achieve suitable local administration, maintenance at the site of implantation or target site, and sustained therapeutic dosage. Therefore, to overcome the challenges and to maintain the effectiveness (e.g. its bioactivity) of a particular therapeutic drug, it necessary to consider the properties of the carrier [51].

Hydrogels are considered to be an effective carrier for the delivery of therapeutics. Hydrogels are defined as a three-dimensional, hydrophilic network of polymer which are bonded by covalent, electrostatic interactions, van der Waals forces or physically assembled into infinite network [53]. It is made up of a large percentage of water, and is thus highly permeable allowing the transport of biomolecules and nutrients mimicking hydrophilic tissue [54]. When compared to a biological tissue, there is a similarity in the water content of the hydrogels. For these reasons it can be used for tissue engineering applications. Hydrogels are considered a versatile material for local non-invasive drug delivery as well as for cell encapsulation [55]. Depending upon the need hydrogels, can be designed which can deliver drugs through bulk erosion where the hydrogels are degraded when water penetrates in its network. Alternatively, the drugs can be delivered through surface erosion where the hydrogels are degraded hydrolytically, thereby delivering the therapeutics [52]. In addition to drug delivery capabilities, some hydrogel carriers are capable of achieving cell delivery and may facilitate cell proliferation and differentiation with low levels of toxicity [52].

I.9. Potential rhBMP-2 Carrier: Keratin Biomaterial

The success or the failure of graft incorporation depends on various physiological properties of the bone grafts. These properties are osteogenesis, osteoinduction, and osteoconduction. Osteogenesis is the natural bone growth or bone repair process. It is the ability of the graft to produce new bone by mineralization and calcification of the collagen matrix. This property is found in fresh autogenous bone and in bone marrow cells [6]. Osteogenic graft material contains viable osteoprogenitor cells or the potential viable cells that can differentiate to inducible osteogenic precursor cells. Osteoconduction provides a surface that permits new bone growth, thereby aiding in bone healing process. Osteoconductive grafts lead to the formation of interconnected network and intrusion of osteogenic precursor cell to the graft site. Osteoinduction is process by which stem cells or progenitor cells are stimulated by growth factors to differentiate to form mature bone cells. Demineralized bone matrix (DMBs) and bone morphogenetic proteins (BMPs) contains are examples of osteoinductive matreials or molecules, respectively [26].

As discussed above, an ideal bone graft should have an osteoconductive structure along with the ability to deliver osteogenic growth factors to support implanted or recruit the host's osteogenic cells. A biomaterial can be considered as a potential carrier that can replace a tissue or a particular function in the body and should be biocompatible, biodegradable and be able to provide mechanical stability [56]. Many natural polymers such as collagen [57], elastin [58], gelatin, fibronectin [59], aliginate [60], silk fibroin [61], chitosan [62] and hyaluronic acid [63] have been studied as potential carriers of rhBMP-2 for bone regeneration due to their ability to behave as an extracellular matrix (ECM)-like scaffold [12].

The carrier system that is the focus of this thesis is keratin, which is a proteinaceous biomaterial. Keratin is a natural polymer that belongs to a family of structural proteins that have either soft or hard keratins [64]. Soft keratins are cytoskeletal elements found in epithelial tissues while hard keratins can be found in nails, hooves, wool and hair [65], [66]. Human-hair derived keratin is the source material for the work described in this thesis. Figure 3 shows a schematic of a wool fiber which has a structure similar to human hair. Keratin can be divided into three groups: 1. alpha-, 2. beta-, and 3. gamma-. Alpha (α) keratins are the most abundant type of intermediate filament proteins (IFPs) that are found in fiber cortex. It has an average molecular mass of ~ 40-60 kDa with low sulfur content and an α-helical secondary structure [65]. Also, α-keratins have high enough molecular weight to allow the formation of chain entanglements to achieve hydrogel formation. γ-keratins are the matrix or globular protein and have high sulfur content with molecular mass of ~ 11-26 kDa [67]. β-keratin forms the majority of the cuticle and provides protection to keratin fibers from physical and chemical damage. The extraction of β-keratins is challenging and these proteins have not been reconstituted into biomaterial structures, to the best of our knowledge [66].

Keratin biomaterial are the focus of this thesis as a carrier of rhBMP-2 for a number of reasons. Keratins have been shown to have minimal *in vivo* toxicity [8], [9]. Keratins are capable of promoting cell attachment and growth due to the presence of cell adhesion sequences including arginine-glycine-aspartic acid (RGD) and leucine-aspartic acid-valine (LDV) [68], [69], thus showing similar features to extracellular matrix (ECM) proteins such as collagen and fibronectin [70]. Also, the properties of keratins allow it to be fabricated and delivered in various

forms. These include fibers, films, hydrogels or to be formed into so-called scaffold forms (e.g., as freeze-dried sponges).

The chemical properties of the keratin (and the properties of biomaterials such as hydrogels made from them) depend on the extraction process used to obtain them. The key feature related to this is the presence of the large number of cysteine residues found in keratin and the ability to form disulfide bonds (or not, as described below). Because of their molecular weight, hydrogels of keratin can be prepared with no need for an external cross-linking agent. When the extraction is carried out by using oxidation reaction, cysteine is converted to cysteic acid derivatives, referred to as "keratose". The oxidizing agent acts very slowly since they are not specific for the disulfide groups. Oxidants that are reported in the literature include peracetic acid, bromine, permanganate and hydrogen peroxide [71]. Keratose forms of keratin are hygroscopic, water soluble, susceptible to hydrolytic degradation at extremes of pH due to polarization of the backbone caused by the electron withdrawing properties of the cysteic acid. Also, due to the presence of cysteic acid it is unable to form disulfide bonds with the neighboring polymer chain and hence degrades within weeks in vivo and in vitro [13].

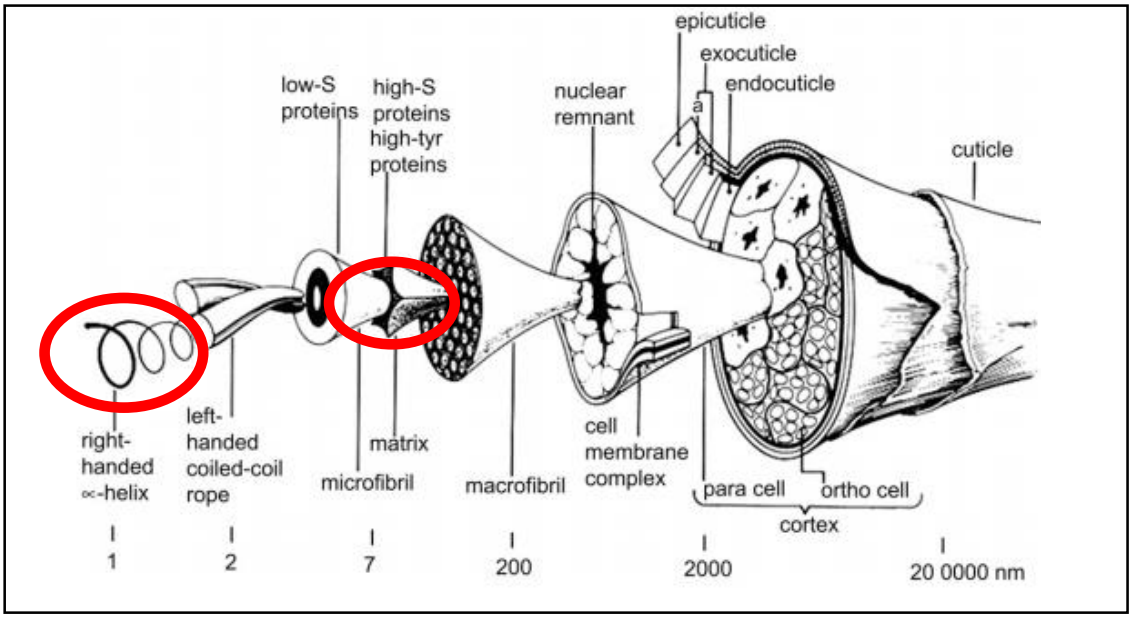

Figure 3: Schematic of a wool fiber drawn by Bruce Fraser and Tom MacRae that has a resemblance -with superstructure of human hair [8]. Used with permission.

Kerateines are extracted by using reductive extraction conditions, and the cysteine groups have thiol groups preserved that can ultimately form disulfide bonds. This property makes kerateine less susceptible to hydrolytic degradation and it can therefore persist in its hydrogel or scaffold form for several months *in vivo* and *in vitro*, depending on the initial concentration [66]. Reducing agents act quickly in the extraction process compared to oxidants. Thioglycolic acid [10], potassium cyanide, sodium sulfide[11], and sodium sulfite[12] are reducing agents reported for the extraction of kerateines [13]. Kerateines are more stable at extremes of pH, less polar and slightly less soluble in water [65]. Therefore, in order to obtain a successful keratin carrier, the cysteine groups on the keratin should be modified to obtain a desirable level of disulfide bond that will aid in the sustain release of rhBMP-2.

Previous studies conducted by members of our research team indicate that the rate of degradation of keratin hydrogels can be controlled by the manipulation of the sulfur groups in the cysteine residues of keratin. Alkylation of the cysteine group by iodoacetamide (IAM) leads to the modification of keratin hydrogels by decreasing the free thiol content of kerateine. The result from the study indicated that the degradation rate of modified kerateine (MKTN) was more rapid than KTN due to low level of disulfide crosslink. While the degradation rate of MKTN was slower than the KOS hydrogels, thus indicating that the rate of hydrogel erosion can be controlled by manipulating the cysteine residue in the keratin hydrogels [72].

In a separate study by members of our team, keratose or kerateine have been used to achieve control over the release of several growth factors. The release of basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and insulin-like growth factor 1 (IGF-1) strongly correlated with the degradation rate of keratin. Thus bFGF, VEGF, and IGF-1 were released quickly from keratose hydrogels and slowly from kerateine hydrogels. It has been noted, however, that both properties of the keratin hydrogel materials as well as physiochemical properties like molecular weight and isoelectric points of the drug or growth factor are responsible for the release of a particular drug/growth factor [73].

These previous studies describe an approach to modulate disulfide crosslinks with an external agent (iodoacetamide alkylation) or the ability to control release only with KOS or KTN as the carrier. However, the simple approach of using combinations of KOS and KTN to deliver

growth factor/drug in a tunable fashion is not described in the peer-reviewed literature. We have previously used this simple approach to tune rates of keratin hydrogel degradation [74], but the effects on rhBMP-2 release and bioactivity are unknown.

1.10. Rationale for Thesis

There are ambiguity in the literature whether soluble rhBMP-2 (i.e., rhBMP-2 released from carrier that diffuses to "nearby" cells) or matrix-bound rhBMP-2 (i.e., rhBMP-2 that remains associated with the carrier that interacts with cells infiltrating the scaffold) is principally responsible for its bioactivity[75], [76] . We, therefore, developed our keratin system with tunable rates of degradation (i.e., mixtures of KOS and KTN) that presumably would have variable rates of rhBMP-2 availability. The main objective of this study is to better understand the delivery of rhBMP-2 from keratin hydrogels formulated from mixtures of keratose and kerateine and how this delivery affects the bioactivity of the rhBMP-2. This study should help to provide a better understanding of the means by which rhBMP-2 leads to the formation of bone tissue when delivered from collagen or keratin carriers [9], [11] and for what duration of time these growth factors are active such that it can heal a bone fracture. That is, this work should provide insight into whether the soluble form or matrix-bound form of the BMP-2 is responsible for bone regeneration. Given the use of collagen controls, this should also provide insight into the mechanism of action of the current clinically-approved synthetic bone graft system (Infuse). The objective of the thesis was to identify whether the soluble rhBMP-2 or matrix-bound rhBMP-2 or the combination of both was primarily responsible for the bioactivity of BMP-2.

The overarching hypothesis guiding the work of our research team is that the rate of degradation of the keratin biomaterials controlled by manipulation of disulfide cross-linking levels with mixtures of keratose and kerateine can be used to affect delivery of therapeutic agents such as rhBMP-2. In this thesis work, we have employed these tunable keratin carriers to investigate the bioactivity of rhBMP-2. More specifically, we have investigated whether release of soluble rhBMP-2 from the keratin biomaterials or rhBMP-2 that remains associated with the keratin and primarily responsible for observed bioactivity. This work has implications into the design of rhBMP-2 carrier systems, as it can provide direction into whether materials that promote soluble release of drug or

materials that provide retention of the rhBMP-2 may be most favorable for rhBMP-2 bioactivity.

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CHAPTER II- Determination of the Delivery Mechanism by which rhBMP-2 Delivered from Keratin or Collagen Biomaterials Achieves its Bioactivity

Salma Haque

II.1. Introduction

In Chapter 1, we suggested the potential advantages of keratin as a natural polymeric carrier for rhBMP-2 in the use of synthetic bone grafts. The goal of this thesis is to further explore this material and more specifically to assess its ability to serve as an alternate carrier for delivery of rhBMP-2. Keratin is known for having a high number of cysteine residues that are necessary for the formation of disulfide bonds [1]. Dislufide cross link (-S-S-) refers to the covalent bond that, in the case of keratins, links the sulfur groups of reductively extracted (KTN) molecules (either inter- or intra- molecularly). Different percentage of disulfide cross-links in hydrogels fabricated from keratin proteins can be achieved by properly formulating the hydrogels with keratins extracted by different techniques: oxidative or reductive extraction. Keratose (KOS) is obtained by an oxidative extraction of keratin in which cysteine are in the form of cysteic acid. As such, hydrogels made of KOS degrade rapidly as they are unable to form disulfide crosslinks due to the presence of sulfonic acid on the sulfur atoms of the cysteine residues. On the other hand, kerateines (KTN) are obtained by a reductive extraction process in which the thiol groups are preserved and able to form disulfide bonds leading to slower degradation [2]. Moreover, studies in our lab have shown that the degradation rate of keratin hydrogels can be controlled by modulating levels of disulfide crosslinking through formulating different levels of keratose and kerateine into the hydrogels [3][4]. Also, in humans, no keratinase enzymes are known to be present, potentially slowing the rate of enzymatic degradation of keratin *in vivo* compared to other commonly used materials such as collagen for which more selective enzymes exist. The potential advantages of using keratin-based materials as rhBMP-2 carriers for synthetic bone grafts are its osteoconductive behavior and its ability to mimic the native extracellular matrix (ECM) [5]. Since keratin is biodegradable, non-toxic, and readily available, it is a suitable carrier for the delivery of drugs/growth factors such as rhBMP-2 [6].

In this chapter we report studies related to the mechanism by which rhBMP-2 delivered from keratin hydrogels of KOS and KTN mixtures achieves its bioactivity as well as the duration over which this activity is sustained. Keratin hydrogels were formed by mixing different weight ratios of KOS and KTN to get the modulated percentage of disulfide crosslinks and thus a tunable rate of material degradation. We first assessed the degradation rates of kerateine,

keratose and KOS-KTN mixtures to confirm behavior previously reported by our group [7]. We then studied effects of disulfide crosslinks on its pore structure by scanning electron microscopy (SEM). Finally, it is known that rhBMP-2 achieves its effects on cells through cell-surface receptors and subsequent "outside-in" signaling effects [8]. However, in terms of its delivery from biomaterials such as keratin, it is not well-understood how rhBMP-2 predominately reaches cells to have its effect. It have been reported in literature that both the rapid release of the drug molecule followed by the sustained delivery of the drug molecule [9] is important to attract the cells to differentiate and stimulate the process of bone tissue regeneration [10] That is, it is not known whether rhBMP-2 released as soluble drug from the materials diffuses to cells to achieve its effect (soluble effect), if rhBMP-2 remaining on the material achieves cellular interactions (matrix-associated effect), or if it is some combination of these two mechanisms. Therefore, we studied the mechanism and duration of rhBMP-2 bioactivity when it is released from different percentage of disulfide crosslinks hydrogels by using several different in vitro and in vivo methods. In addition, we also explored how collagen achieves its effects as this is the material used for commercial, clinical synthetic bone grafts that involve the delivery of rhBMP-2 (see Chapter 1). Previous studies conducted by our team have shown that both KOS and KTN individually are suitable carriers for rhBMP-2 to achieve bone regeneration in two different animal models (rat femur defect and rat mandibular defect) [11], [12], and this work may allow for more highly tunable forms of keratin carriers to be used for rhBMP-2 delivery. In total, we assessed release and bioactivity rhBMP-2 from five different formulations of keratin hydrogels based upon their disulfide crosslinking in order to improve future delivery strategies with keratin or other carriers. More specifically, results of these studies might indicate whether rhBMP-2 carriers should emphasis retention at the defect site (matrix-associated delivery) or release of the rhBMP-2 (soluble delivery).

II.2. Materials and Methods

II.2.1 Formation of Keratin Hydrogels using mixtures of Keratose and Kerateine

II.2.1.1. Keratin hydrogel fabrication

Keratose (KOS) and kerateine (KTN) powders were purchased from KeraNetics, LLC (Winston-Salem, NC) in lyophilized and sterile (via 2 MRad gamma irradiation) form. All the experiments were carried out using alpha fractions of the keratin (α- keratin). 10 or 15% w/v gels of keratin were used for these experiments. These gels were prepared by weighing out dry keratin powder (e.g., 150 mg total keratin for 1 mL of gel at 15% w/v). First, the keratin powder was placed in a 15 ml conical tube. 1 ml of water or water containing rhBMP-2 was then added. The experiments were carried out using five different formulations of keratin hydrogels with KOS: KTN ratios as follows: 100:0, 70:30, 50:50, 70:30 and 100:0, resulting in variation in disulfide cross links. The mixtures were thoroughly mixed by agitating manually (by spatula) followed by vortexing. These mixtures were then centrifuged up to the speed of 1500 rpm and then slowed it back slowly. The keratin material was then placed into a 1 mL syringe, and known volumes/masses were placed in 1.5 mL tubes. Then, these mixtures were incubated overnight at 37^{o} C to spontaneously gel.

II.2.1.2. Degradation of Keratin Hydrogels

We assessed the hydrogel degradation profile of KOS, KTN and three other formulations (i.e., 30, 50 and 70% disulfide crosslinking). Keratin hydrogels (10% w/v) were fabricated with rhBMP-2 at the concentration of 100 µg/ml with the method described above. Control keratin gels without rhBMP-2 were prepared by using water to form the gels. Approximately, 100 µL of keratin in water were ejected from the 1 mL syringe into 1.5 mL tubes and were allowed to gel overnight by incubating them at 37^{o}C. Each of the hydrogels were made in triplicate (n=3) and were incubated with 150 µL of phosphate-buffered saline (PBS) placed on top. The PBS was collected at the following time points: 1.5, 3 6, 12, 24, 48, 72, 96, 120, and 144 hours. Fresh PBS was then placed onto the samples. The collected sample were stored at -20^{o}C until analysis was performed. The concentrations of keratin eluting into the PBS was determined by DC Protein Assay (Bio-Rad, Hercules, CA) for each KOS:KTN formulation. This allowed

determination of the amount of soluble keratin being released and, thus, the rate of degradation of the hydrogels.

II.2.1.3. Release of rhBMP-2 from Keratin Hydrogels

For these studies, rhBMP-2 (Medtronic, Mineapolis, MN) molecules were tagged with Alexa Fluor 555-succinimidly ester (AF555, Invitrogen) in order to detect the release of rhBMP-2 by fluorescence. rhBMP- 2 powder was solubilized in water, dialyzed against water (molecular weight cutoff ~ 6-8kDa) to remove the Tris-group from the buffer and then reacted it with AF-555 to obtain the AF-555-tagged rhBMP-2 molecules. The labeling ratio of dye: rhBMP-2 was 1:10.

To assess the release rates of recombinant human BMP-2 (rhBMP-2) from five formulation of keratin hydrogels with 0, 30, 50, 70 and 100% disulfide cross links (100:0, 70:30, 50:50, 30:70, and 0:100 KOS:KTN ratios) which were obtained by mixing KOS and KTN that were loaded with either 10μg or 0μg of BMP-2 (where the 0μg is the control for just the carrier degradation in the absence of rhBMP-2). 10% (w/v) keratin hydrogels were fabricated with the method described above.

There were three groups (n=3) for each formulation each having volume of 100 μL of hydrogel. The hydrogels were incubated with 150 μl of PBS placed on top, and the PBS was collected and replaced at the following time points: 1.5, 3 6, 12, 24, 48, 72, 96, 120,144 hours. The collected sample were stored at -20oC until analysis was performed. Fluorescence of AF-555 rhBMP-2 was measured at 530/25 and 590/35 nm for excitation and emission in a Biotek Synergy HT microwell plate reader (where 25 mm and 35 mm refers to the band width of the band filter on a fluorescent plate reader). The readings obtained were compared with standard curve to determine the concentration of rhBMP-2 released from keratin hydrogel, which was converted to a mass and percent release based on the volume of the PBS (150 μL) into which the rhBMP-2 was released.

II.2.1.4. Pore architecture of Keratin Hydrogels

To determine if the KOS:KTN formulations had the same pore structures, we studied the structure of the five different formulations of keratin hydrogels by scanning electron microscopy (SEM). 15% (w/v) gels were made with water using the method described above. The gels were incubated overnight at 37^{o}C followed by overnight incubation at -80^{o}C and freeze drying in a Labconco Freezone (Kansas City, MO) 4.5L unit. For SEM imaging, the lyophilized hydrogels were cut horizontally in half with a scalpel blade to reveal the inner pore structure. The dried hydrogels were then mounted onto SEM stubs and immediately sputter-coated with gold for 30 seconds at 45 mV in a sputter coater (Denton Desk II Cold Sputter Coater, Moorestown, NJ). The dried hydrogels were then imaged at 500X magnification with a Zeiss Supra 34VP FEG SEM (Oberkochen, Germany) at 4kV at the Miami University Center for Applied Microscopy and Imaging (CAMI).

II.2.2. Determination of the bioactivity of soluble and matrix-bound BMP-2

II.2.2.1. Preparation of Keratin Hydrogels for rhBMP-2 Bioactivity Assays

In order to assess whether rhBMP-2 has its effect as soluble drug or as matrix-bound drug, we prepared keratin hydrogels for subsequent *in vitro* and *in vivo* experiments. 15% (w/v) keratin hydrogels with 100 μg/mL of BMP-2 were prepared at KOS: KTN ratios of 100:0, 70:30, 50:50, 30:70, and 0:100 to control levels of disulfide cross links as described above. Control hydrogels not loaded with rhBMP-2 but with all five different keratin formulations of disulfide crosslinks were used as negative controls. Absorbable bovine collagen sponges (Medtronic, Minneapolis, MN) were used as positive control because they are used in current clinical, FDA-approved synthetic bone graft products. Keratin hydrogels or collagen scaffolds were incubated with 600 μl of PBS and was collected at the following time points: 1, 3 5 and 7 days. After incubation with sterile PBS, the keratin hydrogel or collagen sponge samples were collected at the following time points: 1, 3, 5 and 7 days. Samples of the collected PBS and hydrogels were then at -80^{o}C and stored until time of the analysis for soluble and matrix-bound rhBMP-2 bioactivity.

II.2.2.2. Measurement of Soluble BMP-2 Bioactivity by In Vitro MC3T3 Model of Alkaline Phosphate Production:

In order to assess the bioactivity of soluble (released) rhBMP-2 we developed an assay based on alkaline phosphatase production. Alkaline phosphatase is a marker for the formation of mature osteoblasts that leads to bone formation and was therefore a good marker of rhBMP-2 bioactivity. The rhBMP-2 released from keratin hydrogels into PBS was tested for its bioactivity. Bioactivity was determined by an alkaline phosphatase (ALP) fluorometric assay kit (Bio Vision, Milpitas, CA) by using MC3TC-E1 pre-osteoblasts (American Type Culture Collection, Manassas, VA) between passages 7-12. The MC3T3-E1 cells were cultured in α-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Life Technologies, Carlsbad, CA). These cells were grown inside a cell culture incubator in a humidified atmosphere with 5% CO2 at 37 ^{o} C. The medium was changed every 2-3 days until the desired confluency (70 – 80%) was achieved. For sub-culturing, cells were rinsed with phosphate buffer solution (PBS) and detached by trypsinization with 0.25% trypsin and incubating them for 5 minutes. Trypsin was deactivated by using 4 times the volume of media with 10 % Fetal Bovine Serum (FBS). 10,000 cells were seeded per well of 96 well plates. These cells were incubated at 37^{o}C overnight and then rhBMP-2 from the samples in PBS (collected as described above) were added to MC3T3 cells that are cultured in 96 well plates. For standard curves, known amount of 8 different concentration of rhBMP-2 was added starting at 2000ng/ml. After 3 days in culture with no media change, the cells were rinsed with PBS and 1% Triton, followed by addition of ALP assay buffer and incubation with 4-Methylumbelliferyl phosphate disodium salt (MUP) as substrate for 30 minutes. Then the stop solution from the ALP assay kit (Milpitas, CA) was added and the solutions were read for intensity of fluorescent signal at 360nm excitation and 440nm emission using a fluorescence Biotek Synergy HT microwell plate reader (Winooski, VT) and compared with a standard curve of known rhBMP-2 concentrations to determine the ALP activity. All the experiments were conducted in triplicate (n = 3).

II.2.2.3. Measurement of matrix bound BMP-2 bioactivity

We investigated two different methods to assess the bioactivity of rhBMP-2 associated with the keratin (or collagen) materials. The first was an *in vivo* assay for the formation of bone as quantified by micro-CT analysis. The second was the use of an *in vitro* alkaline phosphatase activity similar to that described above for soluble rhBMP-2. We describe each of these approaches below**.**

II.2.2.3.a. In vivo ectopic bone growth assay

Our result for soluble rhBMP-2 indicated low bioactivity (Figure 7), it is possible that rhBMP-2 which is still associated with the matrix of the keratin hydrogels is responsible for promoting bone regeneration. In order to assess the bioactivity of the matrix-bound rhBMP-2, we initially proposed to use an *in vivo* ectopic bone assay. For finding the volume of the ectopic bone growth in unknown sample, we did pilot study which included a known amount of rhBMP-2. All animal procedures were reviewed and approved by Miami University Institutional Animal Care and Use Committee. To perform the bioactivity test, pilot animal study was performed. In this model, mice were injected with different concentration of rhBMP-2 in keratose hydrogels with needle near the soleus of the model. Mice were placed under general anesthesia with isoflurane (3-5% isoflurane to induce and 1-3% on a nosecone to maintain). On the right hind limb, the hair was shaved, rubbed with betadine solution, and washed with 70% ethanol. The scrubbing and washing was repeated 3 times.

Keratin hydrogels with known amounts of rhBMP-2 were used for these initial pilot studies. A syringe was loaded with of 50µl of keratin gel. Four different concentration of rhBMP-2 starting at 500 µg/ml, 250 µg/ml, 100 µg/ml and 0 µg/ml were used, where keratose (KOS) was used as the carrier for the pilot study. The gels were then injected (one per animal) with a sterile gauge needle into the posterior region of the right soleus muscle of the mouse. The animals were allowed to recover from anesthesia and were housed for 4 weeks.

After 4 weeks, animals were transported to the University of Cincinnati's Vontz Imaging Center in micro-isolator cages for µ-CT scans to assess ectopic bone growth. Mice were again anesthetized with isoflurane until the imaging procedure had been performed. The imaging was

performed at 500microAmp current with voltage of ~80kV and exposure of 450ms. 3-D images were reconstructed from a single rotation about the axis for 360 steps to achieve the voxel resolution of ~50 cubic microns. After obtaining the image the animals were returned to Miami University and humanely euthanized. The data obtained were reconstructed into three-dimensional images to allow analysis for the volume of bone using the software Osirix as a measure of the bioactivity of the rhBMP-2 in the keratose hydrogels injected into the mouse model.

II.2.2.3.b. In Vitro Alkaline Phosphatase Activity of Matrix-bound BMP-2

Ectopic bone growth was observed when the dosage of rhBMP-2 was 25μg (500 μg/mL concentration in a 50 μL hydrogel). Each sample in the *in vivo* study contained less than 10μg of rhBMP-2. This limiting detection limit necessitated an alternative assay. We therefore elected to use the alkaline phosphatase assay described above to assess the matrix-bound rhBMP-2 bioactivity, but with some modifications.

In order to assess the bioactivity of rhBMP-2 in unknown samples, a standard curve was made using the known amount of rhBMP-2. We prepared a standard curve for each keratin formulations (and collagen). For the standard curve, the 15% (w/v) keratin hydrogel or a collagen sponge (Infuse, Medtronic) with 8 known amounts of rhBMP-2 were prepared. The experiments were carried out using the same five different formulation of keratin hydrogels described above with differing levels of disulfide crosslinks: KOS (100:0), 30:70 KOS: KTN, 50:50 KOS: KTN, 70:30 KOS: KTN and KTN (0:100). The formulations were thoroughly mixed by agitating manually and then followed by vortexing. These mixtures were then centrifuged briefly and then packed into 1ml syringe, and known volumes/amount were placed in 48 well plates. 200 μl for four formulation (i.e. KOS, 50:50, 30:70 AND KTN) and 100 μl for 70:30 KOS: KTN was used. Then, these mixtures were incubated overnight at 37^{o}C to gel. Five separate standard curves were prepared – one for each KOS: KTN formulation. All the experiments were conducted in triplicate (n = 3).

Bioactivity of 8 known amounts of matrix-bound rhBMP-2 starting at 100 μg/ml as well as the unknown amount of rhBMP-2 in the samples collected at the time points specified for the

soluble rhBMP-2 bioactivity experiments described above were determined by an alkaline phosphatase (ALP) fluorometric assay kit (Bio Vision, Milpitas, CA) by using MC3TC-E1 pre-osteoblasts (American Type Culture Collection, Manassas, VA) between passages 7-13. The MC3T3-E1 cells were cultured in α-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Life Technologies, Carlsbad, CA). These cells were grown inside a cell culture incubator in a humidified atmosphere with 5% CO2 at 37^{o}C. The medium was changed every 2-3 days until the desired confluent state (70-80%) was achieved. Then for sub-culturing, cells were rinsed with phosphate buffer solution (PBS) and detached by trypsinization by using 0.25% trypsin and incubating them for 5 minutes. 50,000 cells per well were seeded into a 48 well plate that had been previously loaded (via syringe) with each keratin hydrogel formulation. In case of 70:30 KOS: KTN, 25000 cells were seeded on the individual well of 48 well plates. The reason for using fewer cells for 70:30 KOS: KTN was that a smaller amount samples/gels were available after the freeze-thaw. The freeze-thaw cycle of the gels led to a loss of hydrogel consistency for unknown reasons, and it was difficult to pack the gels in the syringe resulting in loss of volume. Cells were incubated at 37^{o}C for 3 days with no media change on each of the gels and then collected samples in media were assayed bioactivity by using an ALP kit. For this assay, ALP assay buffer was added to each sample. The samples were then incubated with 4-Methylumbelliferyl phosphate disodium salt (MUP) substrate for 30 minutes. Then the stop solution from the ALP assay kit was added and the solutions were read for fluorescent signal at 360nm excitation and 440nm emission using a fluorescence Biotek Synergy HT microwell plate reader. The readings for each (unknown) sample were compared to the standard curve of known rhBMP-2 concentrations was made for each of the five formulations (see above). All the experiments were conducted in triplicate (n = 3).

II.3 Results

Formation of Keratin Hydrogels using Mixtures of Keratose and Kerateine

II.3.1. Degradation of Keratin Hydrogels

The degradation profiles of different the formulations of kerateine and keratose were tested by DC protein assay in order to confirm previous results from our group that varying levels of disulfide crosslinking affect the rate of degradation. Figure 4 shows the degradation profiles for the various formulations, which indicates that the rate of degradation of KOS hydrogels are more rapid as compared to KTN hydrogels, as expected. The reason for this is believed to be due to absence of disulfide crosslinking in keratose indicating that higher the percentage of disulfide cross link, slower is the degradation of the hydrogels. It can be observed from the result that as the KTN content is increased (i.e., the percent of disulfide bonds are increased) the rate of degradation of the hydrogels decreases.

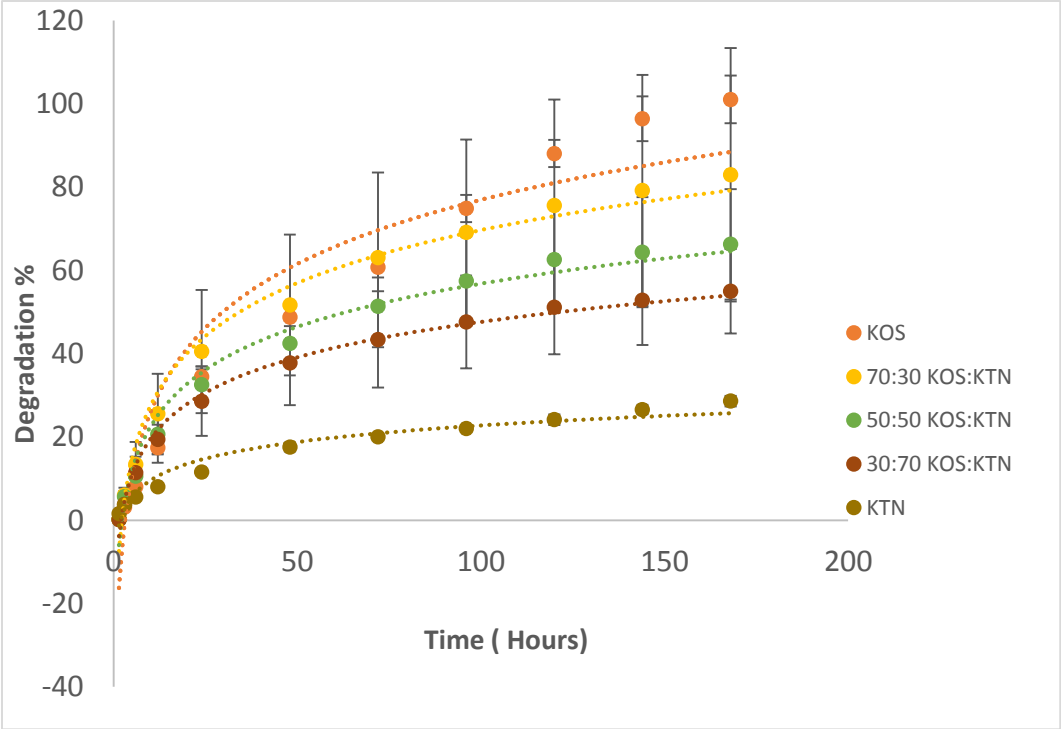

Figure 4. Degradation profile of different formulations of keratin hydrogels (10% w/v) measured by DC protein assay (n=3). Error bars indicate standard deviation.

II.3.2. Release profile of BMP-2 from Keratin Hydrogel

The release profile of rhBMP-2 is shown in Figure 5 for the five different keratin formulations tested. Unexpectedly, the release profiles were similar and no statistical differences were observed for all the formulations of keratin mixture except for the rhBMP-2 released from kerateine hydrogels. Also, it should be noted that the release profile did not follow the degradation pattern. According to the hypothesis the percent of disulfide bonds plays an important role in the degradation of the keratin hydrogels, however the degradation of keratin hydrogel and percent of disulfide crosslinking had no effect in the release profile of the rhBMP-2. It should be noted that other members of our team have recently found that several other growth factors (bFGF, VEGF, and IGF-1) are correlated to keratin degradation rate, but we did not observe this effect with rhBMP-2 [17]. The release profile of rhBMP-2 as shown in the Figure 5, from all five different formulations were quite similar and no noticeable differences were observed. Also, it should be noted that the release profile did not follow the degradation

pattern. According to the hypothesis the percent of disulfide bonds plays an important role in the degradation of the keratin hydrogels, however the degradation of keratin hydrogel and percent of disulfide cross linking had no effect in the release profile of the rhBMP-2. It was also observed that release for rhBMP-2 was characterized by first order release kinetics.

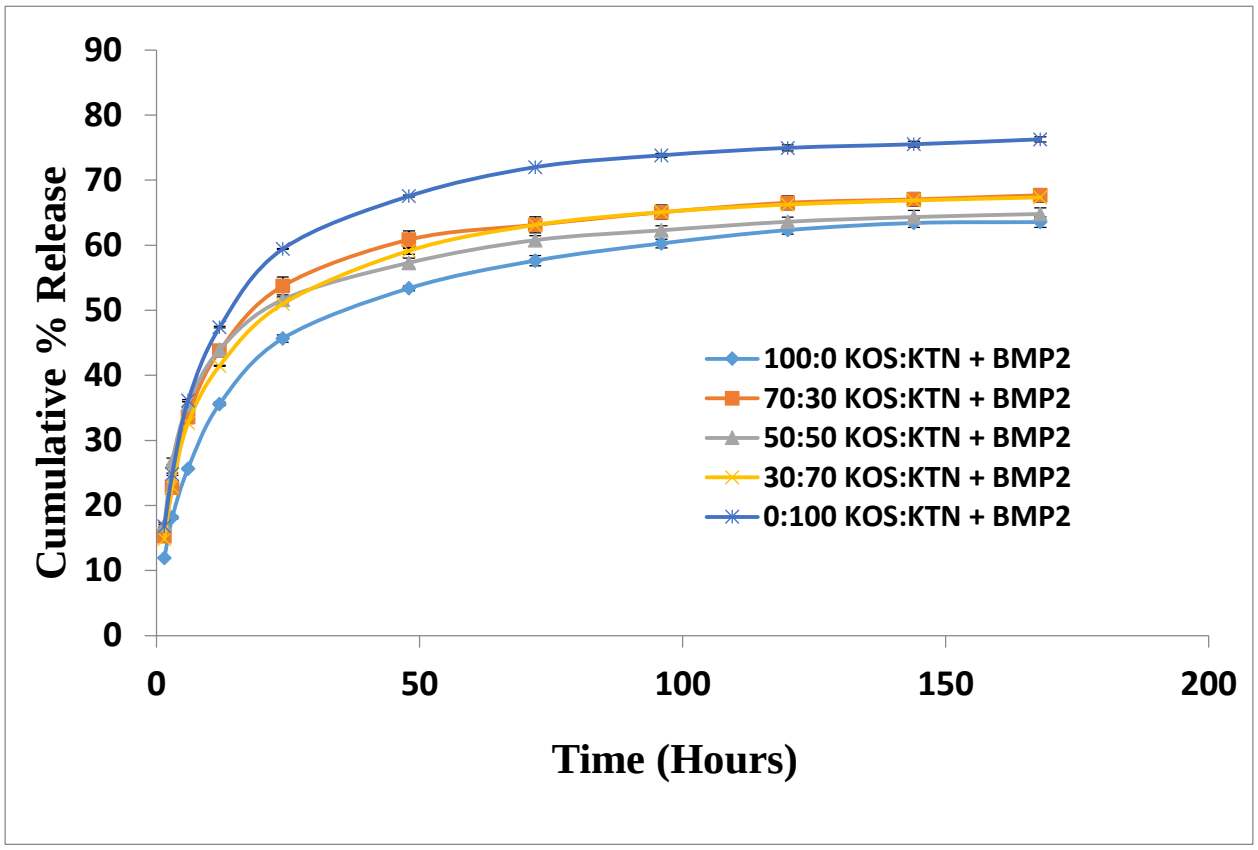

Figure 5: Release profile for 10 µg/ml BMP-2 tagged with Alexa Fluro 555 from five different formulations of keratin hydrogels (10%w/v). The fluorescence was measured at the sensitivity of 55. (n=3). Error bars indicate standard deviation.

II.3.3. Structure of Keratin Hydrogel

It was observed that the gels formed with five different formulations has similar appearances in terms of their pore structures. The freeze dried samples shown in Figure 6 obtained by SEM imaging reveals that all the formulations are porous in nature and have similar pore diameters of approximately 20 µm. Also, mercury porosimetry measurements taken by

members of our laboratory's team showed that the porosities of each formulations to be similar (data not shown).

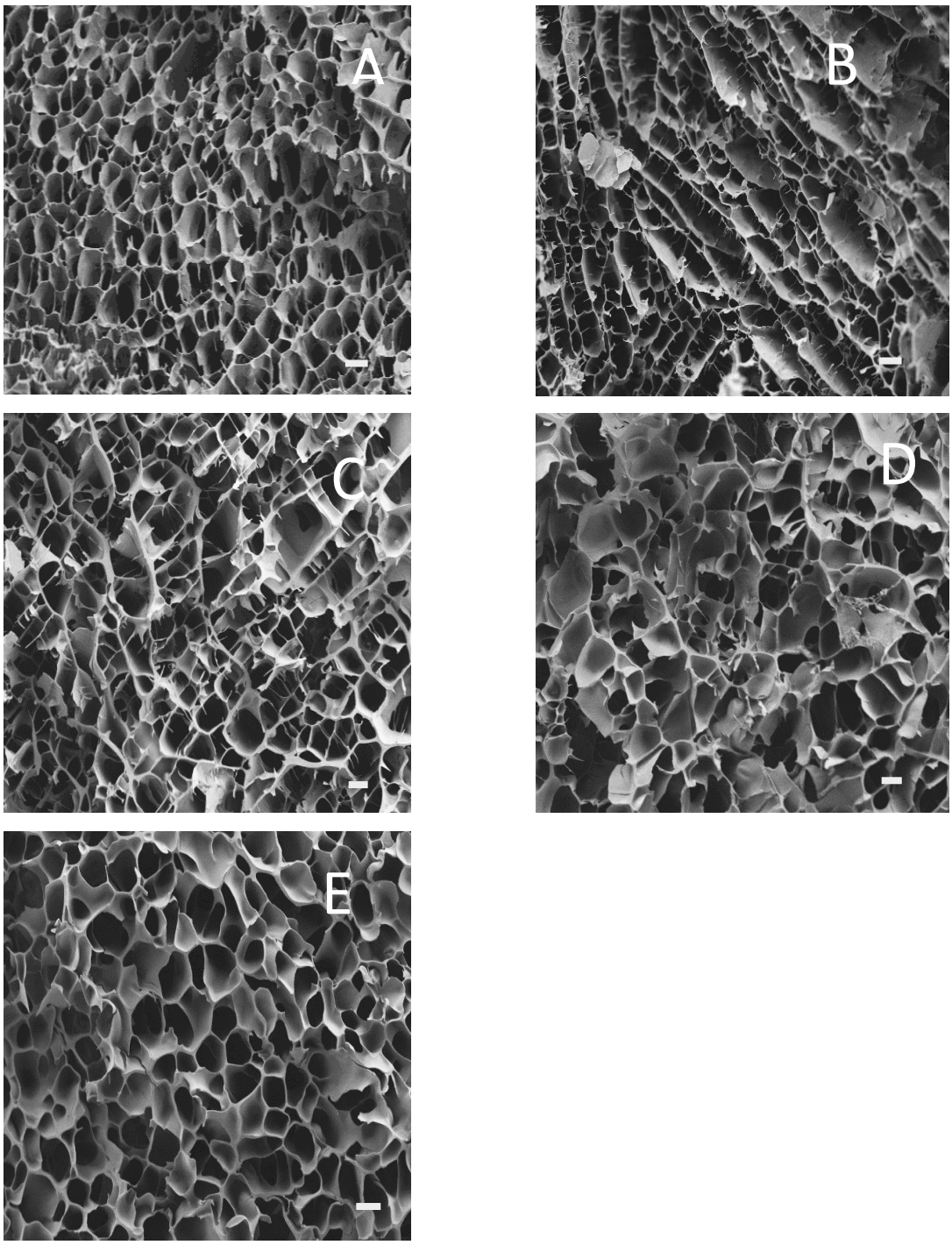

Figure 6. Scanning electron microscope (SEM) images of different formulations of keratin hydrogels (A) KOS (B) 70:30 KOS: KTN (C) 50:50 KOS: KTN (D) 30:70 KOS: KTN (E) KTN illustrating the porous structure of the different weight ratios of keratose and kerateine gels. Scale bar = 20 μm. All images were taken at 500x magnification.

II.3.4. Measurement of soluble BMP-2 bioactivity

Bioactivity of soluble (i.e., released from keratin hydrogels) rhBMP-2 was measured by an *in vitro* model using MC3T3-E1 cells alkaline phosphate production as an outcome measure. A quantitative fluorometric ALP assay was used that detects the production of alkaline phosphatase enzyme produced by the MC3T3-E1 pre-osteoblasts cells when stimulated by rhBMP-2. As shown in Figure 7 bioactivity observed for soluble rhBMP-2 is very low for all five different formulation of hydrogels. In comparing the bioactivity to the amount of rhBMP-2 release shown in Figure 5 where quantities of release are in micrograms/mL, less than 1% was bioactive as indicated by the measurements in ng/mL. Although direct comparison is not possible due to differences in the initial amount of rhBMP-2 present in each formulation (see Chapter 3 for further discussion) it seems apparent that levels of soluble rhBMP-2 bioactivity were very low. Of all the formulations kerateine showed the highest bioactivity when soluble rhBMP-2 released from the hydrogels was assayed. Statistical analysis on the data revealed that there was no significant differences in the bioactivity of collagen and the keratin formulations at day 1, but that collagen was statistically different than KTN at day 3 and all keratin formulations at day 5. There was a trend of increasing bioactivity in the keratin formulations containing increasing levels of KTN, particularly by day 5, and full statistical comparisons are provided in the figure/figure caption.

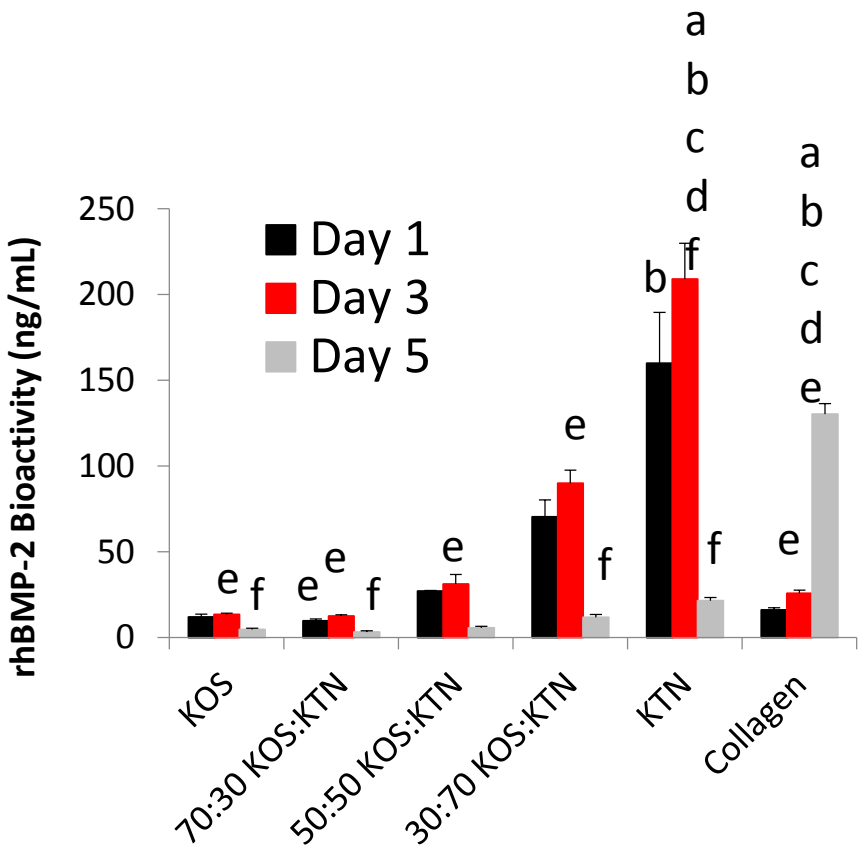

Figure 7. Bioactivity of soluble BMP-2 bioactivity with respect to time for different formulations of keratin hydrogels (15% w/v) and collagen measured by using Alkaline phosphatase fluorometric assay. The fluorescence was measured at the sensitivity of 35. (n=3). Error bars indicates standard deviation. 'a' indicates $p<0.05$ Vs KOS, 'b' indicates $p<0.05$ Vs 70: 30 KOS: KTN, 'c' indicates $p<0.05$ Vs 50: 50 KOS: KTN, 'd' indicates $p<0.05$ Vs 30: 70 KOS: KTN, 'e' indicates $p<0.05$ Vs KTN and 'f' indicates $p<0.05$ Vs collagen, Tukey's test.

II.3.5. Measurement of matrix bound rhBMP-2 bioactivity

In our initial effort to assess the bioactivity of rhBMP-2 associated with the matrix of keratin hydrogels, we attempted to use *in vivo* model of ectopic bone growth. As shown in Figure 8(A), the ectopic bone growth was formed where 25 µg of BMP-2 (that is, 50 µL of 500 µg/ml of rhBMP-2) was injected. The animal model where the following concentration of BMP-2: 12.5 µg, 5 µg and 0 µg (that is, 50 µL of 250 µg/ml, 50 µL of 100 µg/ml, and 50 µL of 0 µg/ml) was used no ectopic bone growth was observed as shown in Figure 8. (B), (C) and (D). It should be noted that 25 µg of BMP-2 was essentially used as a positive control to promote growth. Any

assay used to assess the bioactivity of our keratin gel formulations would need to be able to detect activity at less than 100 µg/ml rhBMP-2 because this was the concentration of rhBMP-2 in our keratin formulations. We note that this dosage was used as a past study by our team suggested that this dosage of rhBMP-2 can promote bone regeneration [10] and a planned future study in a different model will also use this concentration. However, over time, rhBMP-2 is known to be released from the keratin hydrogels (see Figure 5), so the actual amount of rhBMP-2 in the gels after that time will actually be lower for the gels since we are assaying for bioactivity at later time points (i.e., after periods of in vitro incubation). Although a previous study showed that limits of detection were higher than required for purposes of this assay [16], we had thought that it might be possible to detect bioactivity by ectopic bone (or cartilage) growth by threshholding the resulting 3-D imaging differently. As shown in Figure 8, this was not possible. Thus, although the results are consistent with those previously reported, this *in vivo* assay did not provide a low enough limit of detection to assess bioactivity of rhBMP-2 remaining associated with keratin hydrogels. Because, this *in vivo* method did not work we elected to investigate the use of an *in vitro* assay to assess bioactivity of rhBMP-2 associated with keratin hydrogels. We do note that an *in vivo* assay similar to the one described may allow assessment of rhBMP-2 bioactivity, but not via the µ-CT scan method used here. Rather, a histological evaluation of alkaline phosphatase or other markers of ectopic bone formation would be necessary. These studies may be conducted in the future, and we expect that this approach would allow an *in vivo* approach to be successfully applied.

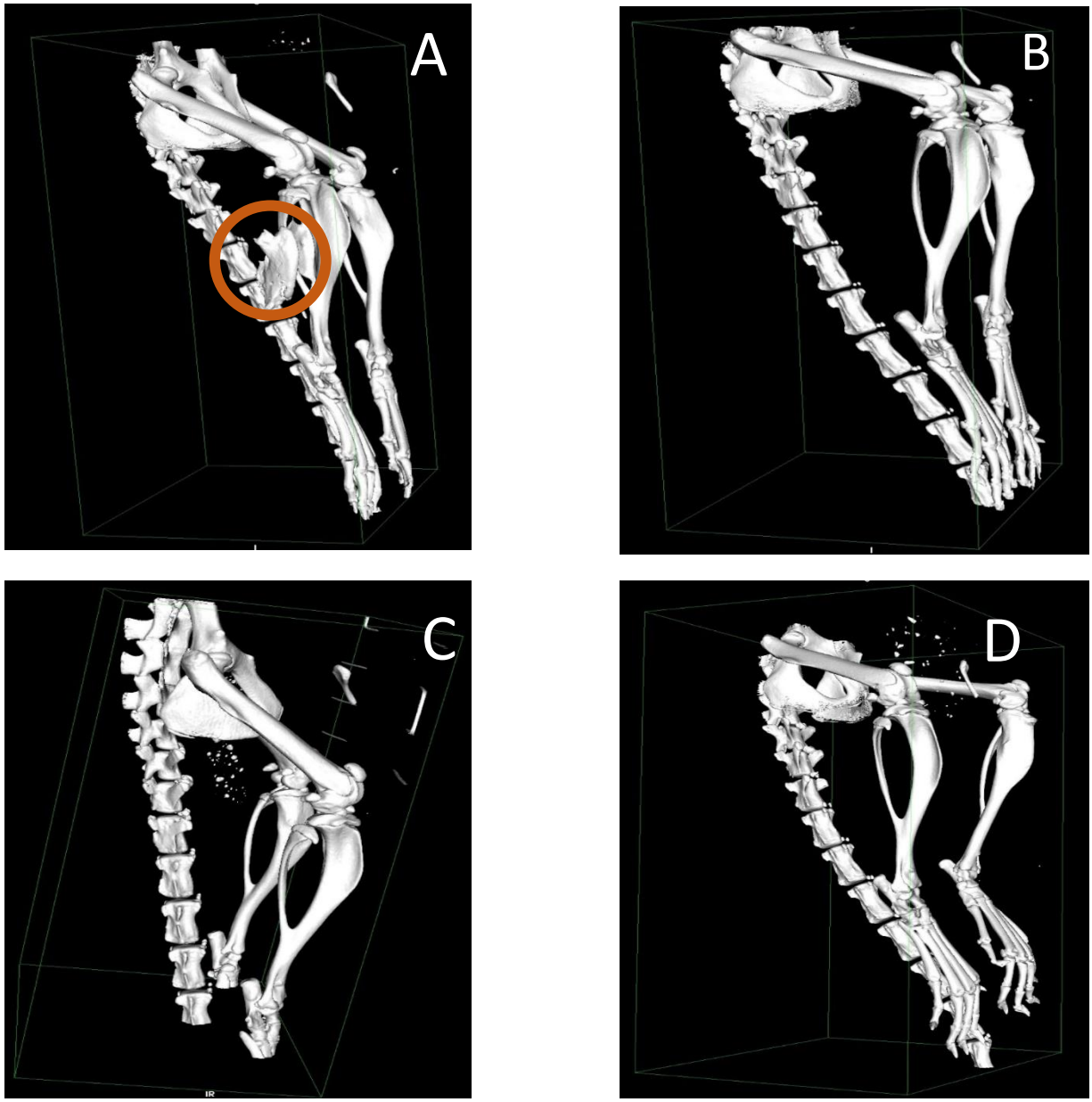

Figure 8. 3D reconstruction of µ-CT images that shows the effect of the dose of BMP-2 delivered from keratose hydrogel used for inducing ectopic bone growth in the mouse model. (A) 25 µg BMP-2 (B) 12.5 µg BMP-2 (C) 5 µg BMP-2 and (D) 0 µg/ml BMP-2. Circle on image (A) shows ectopic bone formation.

II.3.6. Standard Curve for Matrix-bound BMP-2 for Five Different Formulations of Keratin Hydrogels

In order to assess whether an *in vitro* approach could be used to assess bioactivity of rhBMP-2 associated with keratin hydrogels, we first decided to generate standard curves in which varying amount of rhBMP-2 were incorporated into the 5 keratin hydrogel formulations described above. In these experiments, keratin hydrogels were fabricated with varying concentration of rhBMP-2 starting at 100 μg/ml of BMP-2 and made in 1:2 serial dilutions. MC3T3-E1 cells were then placed on top of the keratin hydrogels and allowed to incubate for 3 days. The sample containing media was collected and an alkaline phosphatase fluorometric assay was performed.

Figures 9-13 show these standard curves for varying amounts of rhBMP-2 for each formulation. It is important to note that unlike the *in vivo* assay described above, this *in vitro* method gave limits of detection that were closer to 10 ug/ml whereas the *in vivo* detection limit was above 100 ug/mL. This indicated that, based on the alkaline phosphates vs. micro-CT outcome measure, this *in vitro* approach actually provided 10-fold greater sensitivity. To measure the bioactivity of matrix-bound rhBMP-2 in the unknown samples, five separate standard graph for five different formulation were made as shown in Figure 9-13. For each formulation, we performed a least squares analysis to relate rhBMP-2 concentration to fluorescence. We note that some of the formulations were better fit with a log-fit while others were best fit by a linear fit. The reasons for this are not clear.

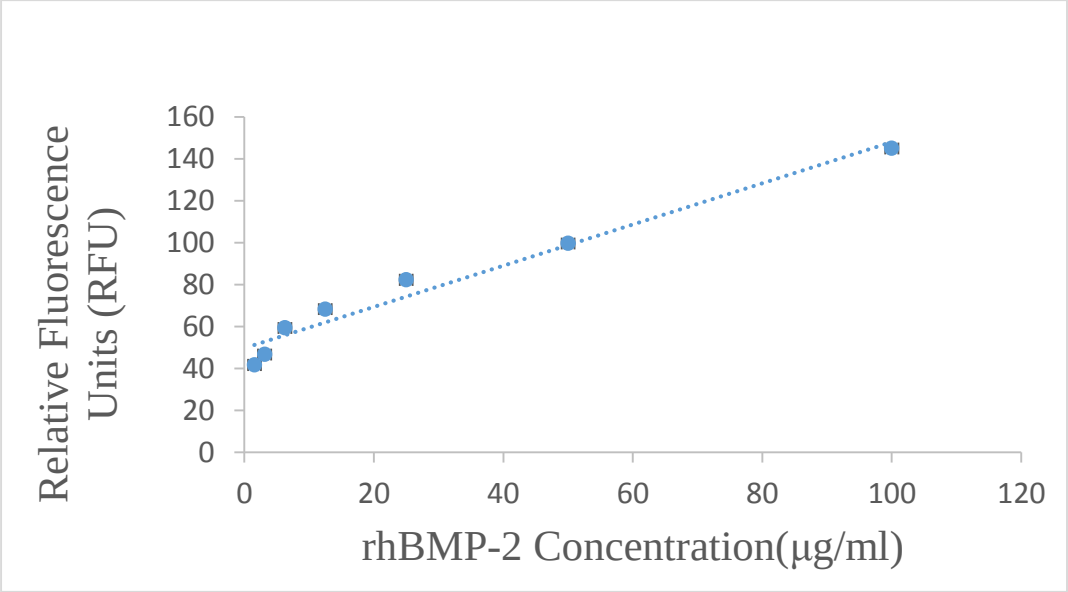

Figure 9. Standard curve for measuring the bioactivity of matrix-bound rhBMP-2 from keratose hydrogels (15% w/v) for 8 known concentration of rhBMP-2 which is measured by using alkaline phosphatase fluorometric assay. Error bars indicate standard deviation. The fluorescence was measured at the sensitivity of 35. (n=3).

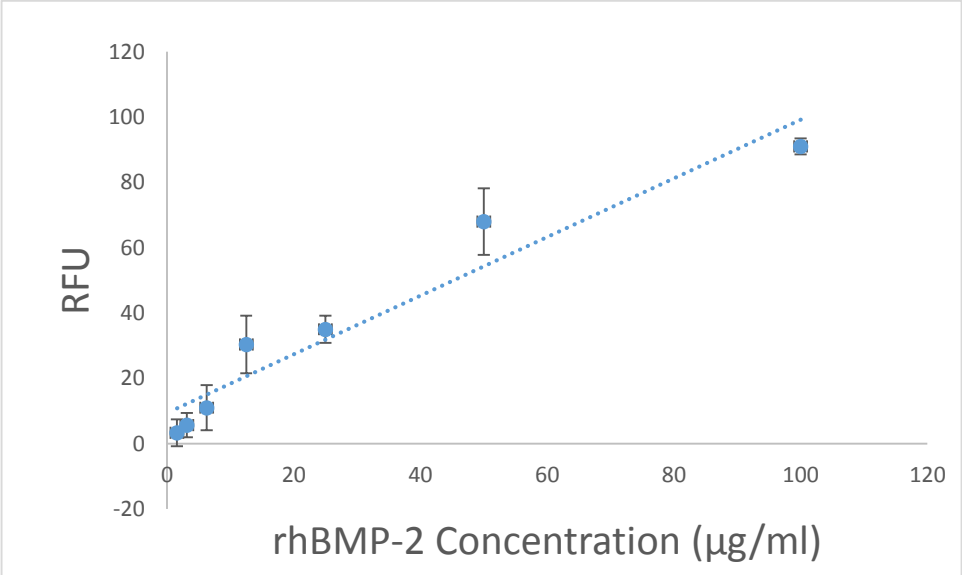

Figure 10. Standard curve for measuring the bioactivity of matrix-bound rhBMP-2 from 70:30 KOS: KTN hydrogels (15% w/v) for 8 known concentration of rhBMP-2 which is measured by using alkaline phosphatase fluorometric assay. Error bars indicate standard deviation. The fluorescence was measured at the sensitivity of 35. (n=3).

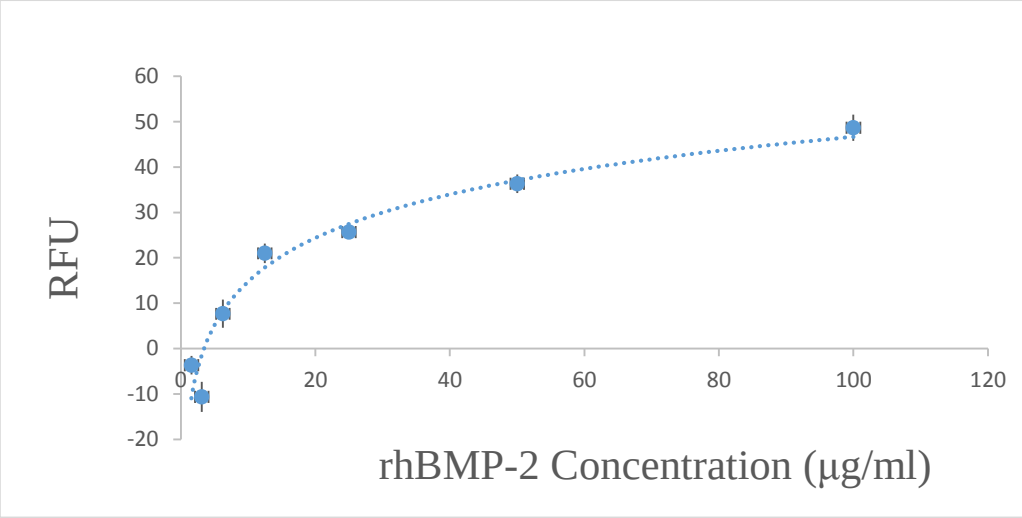

Figure 11. Standard curve for measuring the bioactivity of matrix-bound rhBMP-2 from 50:50 KOS: KTN hydrogels (15% w/v) for 8 known concentration of rhBMP-2 which is measured by using alkaline phosphatase fluorometric assay. Error bars indicate standard deviation. Fluorescence was measured at the sensitivity of 35. (n=3).

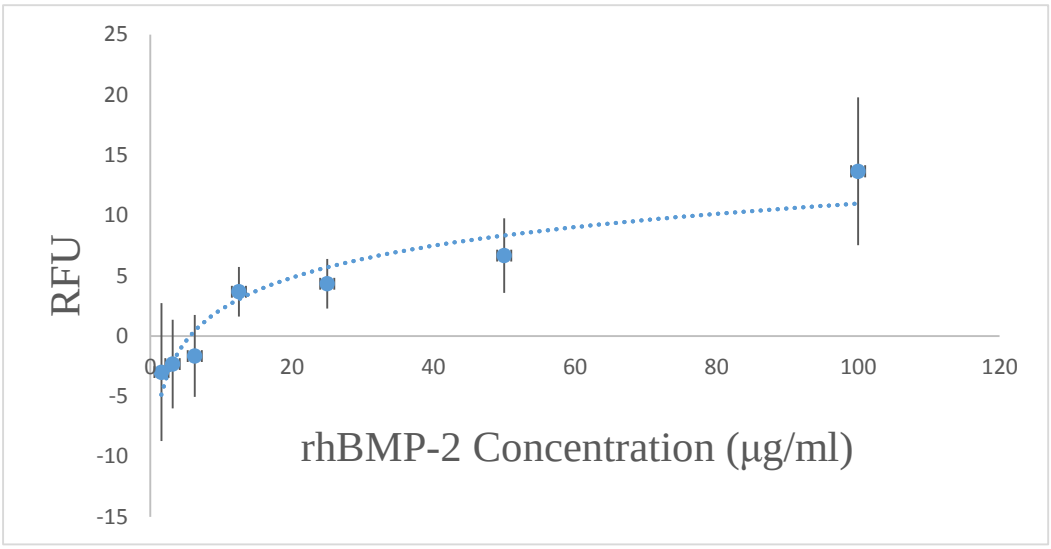

Figure 12. Standard curve for measuring the bioactivity of matrix-bound rhBMP-2 from 30:70 KOS: KTN hydrogels (15% w/v) for 8 known concentration of rhBMP-2 which is measured by using alkaline phosphatase fluorometric assay. Error bars indicate standard deviation. The fluorescence was measured at the sensitivity of 35. (n=3).

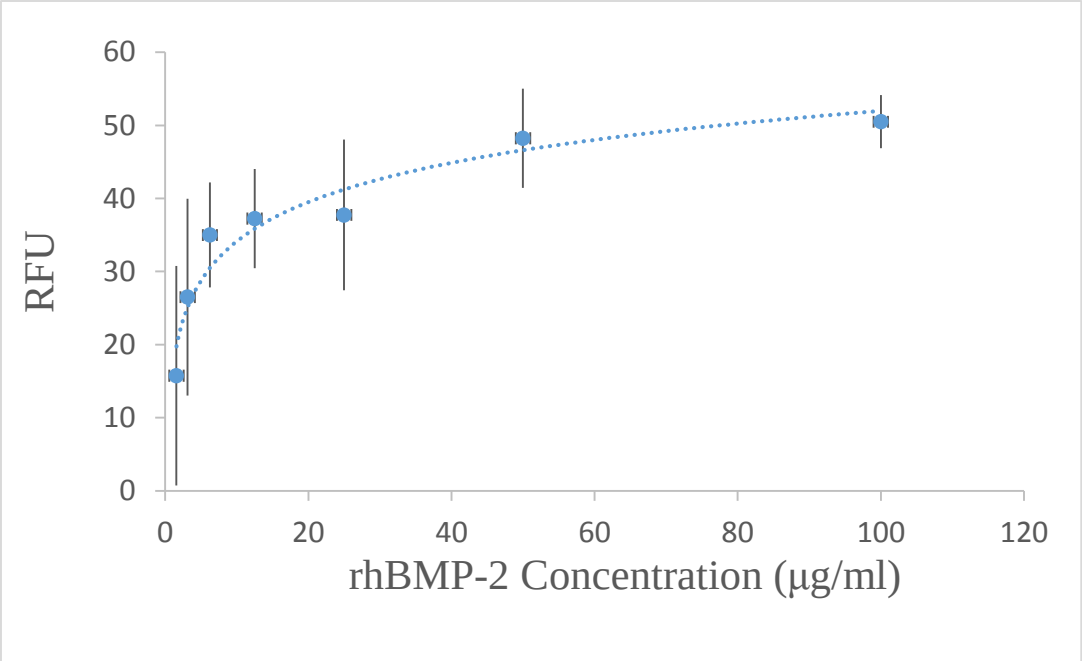

Figure 13. Standard curve for measuring the bioactivity of matrix-bound rhBMP-2 from kerateine hydrogels (15% w/v) for 8 known concentration of rhBMP-2 which is measured by using alkaline phosphatase fluorometric assay. Error bars indicate standard deviation. The fluorescence was measured at the sensitivity of 35. (n=3).

II.3.7. Measurement of matrix bound BMP-2 bioactivity

Because the results of the standard curves indicated limits of detection suitable for our goal of assessing levels of rhBMP-2 activity for rhBMP-2 remaining associated with keratin hydrogels, we elected to proceed with the *in vitro* alkaline phosphatase assay to assess bioactivity of samples with unknown activity that had been cultured for some period of time (1, 3, 5, or 7 days, etc.). Bioactivity of matrix-bound rhBMP-2 was measured by the *in vitro* MC3T3-E1 model using alkaline phosphate assay with MC3T3-E1 cells. It is a quantitative fluorometric assay that detects the production of alkaline phosphatase enzyme which are produced by the MC3T3-E1 pre-osteoblasts cells when stimulated by rhBMP-2. As shown in Figure 14 the percentage of bioactivity observed for matrix-bound BMP-2 is high for KOS, 70:30 KOS: KTN, 50:50 KOS: KTN and 30:70 KOS:KTN as compared to the bioactivity observed for soluble rhBMP-2. This is noted in that soluble rhBMP-2 bioactivity levels were in

the ng/mL range whereas matrix-bound rhBMP-2 was in the microgram/mL range. The bioactivity for KTN was observed to be the lowest. The level of bioactivity observed from different formulations of keratin was significantly different form the collagen at each time point tested. In general, the trend of the keratin formulations was decreasing levels of bioactivity with increasing levels of kerateine in the formulation (increased levels of disulfide crosslinking), and the statistical comparisons are provided in Figure 14 and related caption. Again, although varying levels of bioactivity were seen, all formulations showed increased levels of bioactivity compared to the soluble form of rhBMP-2 shown above (Figure 7). These results are potentially significant in that they suggests that the matrix-bound bioactivity may be responsible for most of the rhBMP-2 bioactivity observed (but see Chapter 3 for further discussion of limitations and drawbacks to this study).

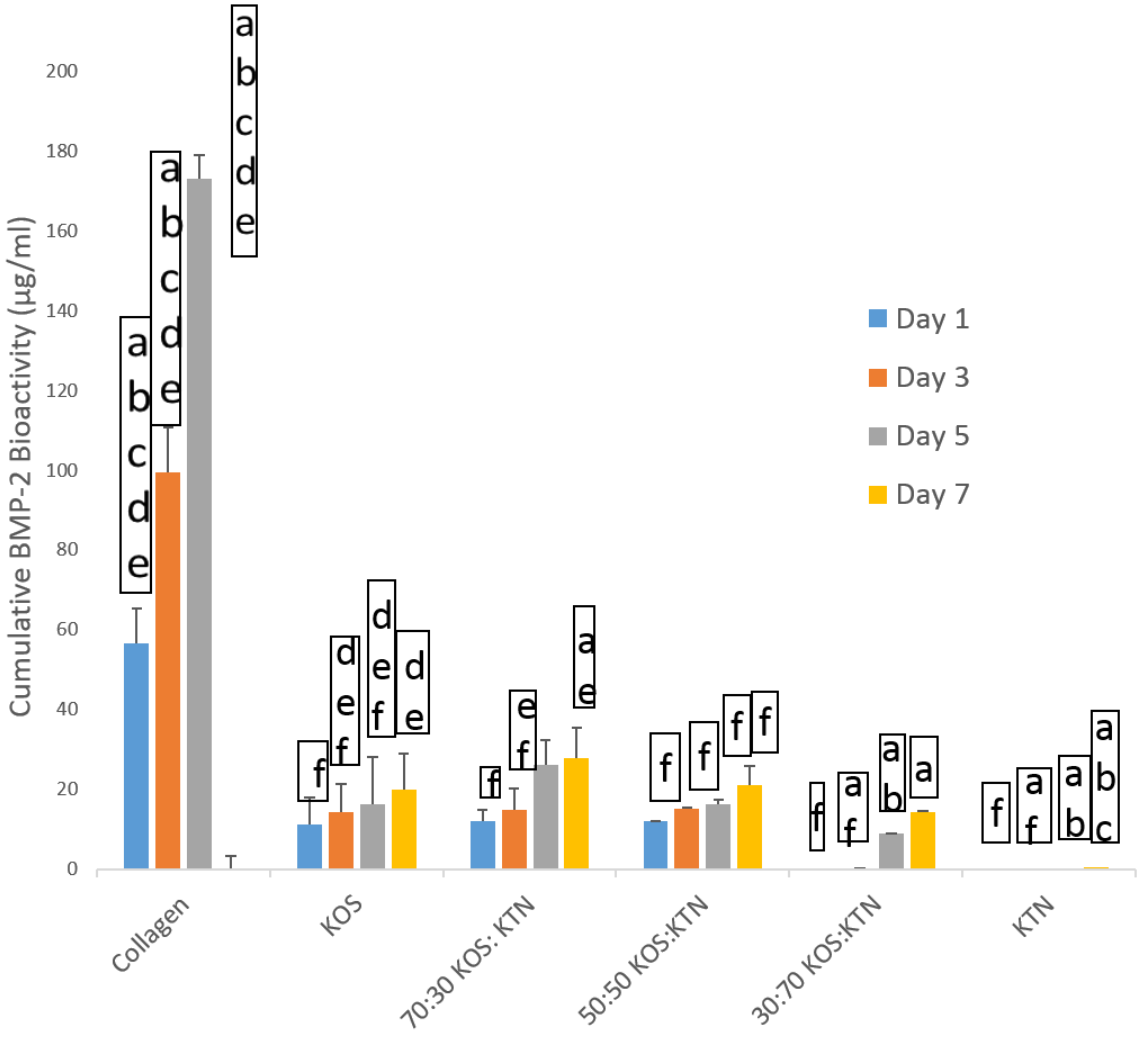

Figure 14. Cumulative bioactivity (µg/ml) of matrix-bound BMP-2 with respect to time for collagen and different formulations of keratin hydrogels (KOS, 70:30 KOS: KTN, 50:50 KOS: KTN, 30:70 KOS: KTN and KTN) (15% w/v) measured by using Alkaline phosphatase fluorometric assay. The fluorescence was measured at the sensitivity of 35. (n=3). Error bars indicate standard deviation. 'a' indicates $p<0.05$ Vs KOS, 'b' indicates $p<0.05$ Vs 70: 30 KOS: KTN, 'c' indicates $p<0.05$ Vs 50: 50 KOS: KTN, 'd' indicates $p<0.05$ Vs 30: 70 KOS: KTN, 'e' indicates $p<0.05$ Vs KTN and 'f' indicates $p<0.05$ Vs collagen, Tukey's.

III. Summary

The studies described were intended to demonstrate the ability of controlled release methods to modulate the bioactivity and subsequent cell response to rhBMP-2. We tuned the properties of the keratin biomaterials by altering the disulfide crosslink in the keratin carrier system by adjusting the ratios of KOS and KTN. In this thesis work, we have generated keratin formulations of different weight ratios that have varying degradation rate depending upon there disulfide crosslinking. We also confirmed the microporous structure of all the different formulations. We then investigated the bioactivity of both soluble rhBMP-2 and matrix-bound rhBMP-2. In Chapter 3, we will provide the discussion of these results.

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CHAPTER III- Discussion of Findings and Future Work

Salma Haque

III.1. Discussion of Findings

The human skeletal system has the innate capability of healing fractures and forming new bone tissue. The disruption of this capability by disease or traumatic injury, however, can lead to bone defects that are unable to spontaneously heal (so-called critically-sized defect). Approximately 15% of fractures exhibit delayed or non-union.

One current solution to treat this problem is the use of an absorbable collagen sponge (ACS; Medtronic's Infuse system) in which recombinant human bone morphogenetic protein 2 (rhBMP-2) is FDA-approved for the treatment of several conditions including lumbar spinal disk fusion, open tibial fracture, and maxillofacial applications [4]. Problems associated with this product are likely due to the use of supra-physiological amount of rhBMP-2 delivered from collagen carriers [5], [6]. One reported problem in off-label applications (e.g., in the cervical spine) of this product is the formation of ectopic bone growth [7], [8].

The objective of this thesis work was to explore keratin biomaterials as a potential alternate carrier for the delivery of rhBMP-2 for bone regeneration. Along with developing the carrier system, it is also important to consider the concentration and timing of delivery of the growth factor. We sought to build on previous studies from our group demonstrating that different percentage of disulfide crosslinking in keratin (controlled by mixing ratios of keratose and kerateine) would affect the degradation rate of the keratin. In turn, we hypothesized that this variation in the rate of degradation of the keratin formulations would affect the release and bioactivity of the rhBMP-2. In these studies, the keratin formulations were compared to resorbable collagen sponges, since this is the current clinical/FDA-approved system. The main emphasis of this thesis work was related to the bioactivity of rhBMP-2 for bone regeneration applications. We also sought to explore the duration of the bioactivity rhBMP-2 and gain insight about the manner by which it reaches cells (i.e., as a soluble drug released from the scaffolds or as a matrix-associated molecule).

We first sought to explore the degradation rate in order to better understand the factors that affect the release of the growth factors and their subsequent bioactivity. A key characteristic of the keratin proteins is the presence of a large number of cysteine residues. The final thiol (and disulfide) content of the extracted keratin powder depends upon the extraction conditions

themselves. Because, KOS (oxidatively extracted keratin) has no disulfide crosslinks as compared to KTN (reductively extracted keratin), we hypothesized that these differences in the disulfide bonds would lead to the tunable degradation rate, along with the release of rhBMP-2 and subsequent bioactivity and duration thereof. The mixing of keratose and kerateine is one method for achieving the tunable degradation and, we suspected, on the release and bioactivity of rhBMP-2. Therefore, in this thesis work we made use of different formulations of keratin mixtures containing varying weight ratios of keratose and kerateine in order to obtain this tunable degradation and investigate subsequent effects on release and bioactivity. More specifically, the work was aimed at attempting to determine whether rhBMP-2 that is incorporated into biomaterials (either collagen or keratin) achieves its bioactivity as a soluble drug (i.e., released from the material) or as a matrix-bound drug in which cells interact with rhBMP-2 still in (or on) the material, or some combination of these two delivery methods. After some initial characterization of the keratin biomaterials, we assessed these key factor (soluble vs. matrix bound) as well as the duration of rhBMP-2 bioactivity (soluble vs. matrix-bound) through the use of *in vitro* alkaline phosphatase production from the MC3T3-E1 pre-osteoblast cells lines.

To better understand the physical properties of the hydrogels resulting from formulations of varying keratose and kerateine ratios, we first investigated the porous architecture of the materials by SEM. The images indicated that all the five formulation of keratin hydrogels were porous in nature (contained interconnected pores; see Figure 6). In addition, all had pore diameters of approximately 20 μm. However, it appears that there are differences in the pore architecture itself. Namely, the mixture formulations appeared to have differences in the wall structure and the pore shapes. We cannot rule out the possibility that the processing (i.e., freezing and freeze-drying) required for SEM led to these changes, but it is possible that the differences in the KOS and KTN ratios are, in fact, responsible for this observation.

We then investigated the degradation rate of different percentage of disulfide crosslink by a DC protein assay (based on the Lowry method). Kerateine contains disulfide cross links in contrast to keratose which contains sulfonic acids and therefore no disulfide crosslinks. As shown in Figure 4, it was observed that KOS degrades rapidly and the degradation rate of KTN was the slowest. The degradation rate of different weight ratios of keratin hydrogels showed that percentage of disulfide crosslink can be used to control the degradation. More specifically, the

keratin hydrogels made with keratin that contains low percentage of disulfide cross link (i.e., more of keratose) were degraded more rapidly compared to those with high percentage of disulfide crosslink (i.e., more of kerateine). This confirms a previous study by our group showing that tunable rates of degradation can be achieved by modulating the KOS:KTN ratio [19]. We are suggesting that it is the related change to the percentage of disulfide cross-link that is responsible for this effect. This result allowed further experiments to be conducted with knowledge that these formulations behave in a manner similar to our previous observations [9].

We next investigated the effect of disulfide cross-link on the release of the rhBMP-2. Previous studies for other drugs and growth factors have shown tunable rates of release of the drug molecule in proportion to the rate of keratin degradation [9]. We expected that the degradation rate of keratin would have similar effect in the release profile of rhBMP-2. However, as shown in Figure 5, we did not achieve nearly the same level of tunability with rhBMP-2 delivery *in vitro* as with these other drugs. We did not find a significant differences in the release of rhBMP-2 from different mixtures of keratin hydrogels and, in fact, unlike other molecules, we observed that rhBMP-2 release was actually the most rapid from the kerateine formulations. The reason for this effect with rhBMP-2 is not clear. We have previously observed that rhBMP-2 seems to have a higher affinity to bind to keratin than other molecules such as recombinant human insulin-like growth factor 1 (rhIGF-1) [9]. In addition, rhBMP-2 has a higher molecular weight than rhIGF-1 and other previously tested molecules. Therefore, it is possible that although formulations with more keratose degrade more rapidly than formulations with more kerateine, the micropores of the gels are still intact enough to retain the rhBMP-2 and that any differences are due to effects that balance diffusional aspects with any binding affinity between the rhBMP-2 and keratin carrier. These results highlight the fact that for sustained release of drug molecules, it is not only the property of the carrier molecule is important but that the physiochemical properties of the drug molecule like molecular weight and isoelectric point plays significant role.

Although this result was unexpected, we elected to explore the rhBMP-2 bioactivity associated with each of the keratin formulations and to compare keratin to collagen. It is known that rhBMP-2 exhibits its bioactivity through interactions with cell surface receptors for the molecule [10]. As noted above, we investigated whether soluble or matrix-bound drug achieves this effect. Our *in vitro* results indicated that the soluble rhBMP-2 had a very low percentage of

bioactivity for all the five different formulations of keratin and also for the collagen sponge. Although kerateine showed higher levels of bioactivity than collagen and the other keratin formulations, it is important to note that all of the formulations tested showed very low levels of bioactivity. That is, it is known that μg/mL quantities of rhBMP-2 are released from the formulations, yet the measured bioactivity levels were in the ng/mL level of activity and thus had fractions of a percentage of the possible level of bioactivity. One drawback to this study is that BMP-2 has short half-life [13],[20]. In the *in vitro* environment in which this study was conducted (PBS without the presence of proteolytic or hydrolytic enzymes), this loss of activity could be due to hydrolysis or denaturation under the presence of phosphate buffer but not due to proteolytic degradation that might be observed *in vivo*. Given that the rhBMP-2 was in solution for several days after it was released from the keratin, in future studies the loss of activity should be normalized to loss of activity in naïve rhBMP-2 of known initial activity to determine loss of activity associated with the presence of rhBMP-2 in the solution itself.

There are two types of delivery reported in literature for rhBMP-2 that has been adsorbed to a surface: rapid release or bolus release [11]. Though burst release is important to stimulate the process and attract the cells to differentiate, sustained release has been suggested as a more favorable scenario to better overall bone regeneration [12]. In terms of the release profile of rhBMP-2 from keratin carrier, it was observed that the release showed both aspects of release: an initial release followed the profile of sustained release, with a release profiles consistent with first order release. Moreover, our bioactivity results seems to suggest that the rhBMP-2 associated with the matrix has substantially more bioactivity than the soluble form of the drug. An important interpretation of the release data in the Figure 5 suggests that the release of the rhBMP-2 is not affected by the keratin formulation (all keratin formulations have similar rhBMP-2 release). Thus, observed differences in the bioactivity may be attributed to the role that the carrier has on preserving the bioactivity before it is released or as it remains associated with the carrier. That is, our interpretation of the soluble rhBMP-2 bioactivity is that the formulations with more kerateine were better able to preserve the bioactivity of the rhBMP-2 before it was released, perhaps by maintaining its structure (i.e., preventing denaturation). Future studies should investigate the structure of the rhBMP-2 after it is released in order to determine if rhBMP-2 released from formulations with more kerateine are in fact maintained in a

non-denatured form. These experiments, however, could be problematic given that less than 1% of the rhBMP-2 from any of the formulations was bioactive at the time points assayed.

In order to assess the bioactivity of the matrix-bound rhBMP-2, we investigated (initially) an *in vivo* approach. Our *in vivo* results showed the formation of ectopic bone growth in the mouse model where 25 µg of BMP-2 was used (500 µg/mL at 50 µL injection volume). Unfortunately, because our release studies use 100 µg/mL in our keratin formulations, this assay was insufficient to detect bioactivity. It should be noted that the dosage of 10 µg of BMP-2 was used as a past study by our team which suggested that this dosage of rhBMP-2 can promote bone regeneration [13], but differences between our previous study and this one may be due to differences in the keratin formulation or the imaging conditions.

We explored the *in vitro* alkaline phosphatase assay in an effort to obtain a lower threshold of detection for matrix-bound rhBMP-2 associated with keratin or collagen materials. We found that this assay provided considerably lower limits of detection than the *in vivo* model, allowing us to better to answer the question about the bioactivity of matrix-bound rhBMP-2. A significant finding of this thesis is that the results of the *in vitro* assay on rhBMP-2 bioactivity indicated that the matrix-bound BMP-2 has a relatively high percentage of rhBMP-2 bioactivity for all the five different formulations of keratin and also on collagen compared to the soluble form of the rhBMP-2. This can be seen by comparing Figure 7 to Figure 14 (*in vitro* alkaline phosphatase activity of soluble rhBMP-2 vs. *in vitro* alkaline phosphatase activity of matrix-bound rhBMP-2), where it is important to note that the soluble drug showed ng/mL levels of bioactivity whereas the matrix-bound values are in µg/mL (~ 100-1000 fold higher than the assays on soluble rhBMP-2). However, it was observed that rhBMP-2 remaining associated with the materials (matrix-bound rhBMP-2) in formulations containing more of the slower degrading kerateine form of keratin did not show as high of level of alkaline phosphatase production over the seven day study. This indicates lower levels of bioactivity from the matrix bound rhBMP-2 released from kereateine hydrogels. We do note that the 70:30 KOS: KTN hydrogel (15%w/v) had a change in its physical characteristics after undergoing a freeze-thaw cycle as it lost its gel consistency. This may indicate effects in the gel formation that we have not investigated, but in terms of these experiments, is noteworthy as it may affect cell access to the rhBMP-2 associated with the material.

An interpretation for the results of the soluble and matrix-bound rhBMP-2 bioactivity is as follows. We suggest that the kerateine preserves the bioactivity of the rhBMP-2. We suspect that this may be because kerateine is more hydrophobic than keratose, thus preventing any hydrolysis of the rhBMP-2 before the rhBMP-2 is released from the carrier and perhaps even immediately after it is released if the rhBMP-2 and keratin remain associated following release for some time. However, we suggest that because the kerateine is more slowly degrading, cells associated with the materials as was done in the matrix-bound experiments may not be able to fully access the rhBMP-2 or degrade the matrix (through non-specific proteolysis by the cells), leading to decreasing levels of bioactivity. That is to say, it is possible that the rhBMP-2 associated with the formulations with higher levels of kerateine would still be bioactive if accessed by cells, but that is not bioactive under the conditions used in these experiments in the sense that it is not accessible by the cells. The method by which cells take up the rhBMP-2 is thus difficult to determine, and the results could remain open to interpretation and future experiments.

It is also important to note that, it was observed that collagen has higher bioactivity of matrix-bound rhBMP-2 compared to all keratin formulations at each time point tested. The immediate conclusion of this result would be that the bioactivity of matrix-bound rhBMP-2 is higher for formulations containing keratose but not kerateine, and that the bioactivity of rhBMP-2 associated with collagen is even higher than both. However, it is important to remember how the rhBMP-2 is actually associated with each material. In the case of collagen, the rhBMP-2 is adsorbed to the surface whereas for keratin it is incorporated into the hydrogel. Thus, it is possible that the cells would have more immediate access to the rhBMP-2 adsorbed to collagen, while the keratin formulations may "protect" or isolate the rhBMP-2 from the cells involved in the assay by a mechanism similar to what was described in the paragraph above as to why kerateine seems to have lower bioactivity than keratose.

In total, the results presented in Chapter II provided insight into the features by which rhBMP-2 exerts its effect. As noted above, there are challenges associated with interpretation of these results. However, it seems apparent that, at the least, matrix-bound rhBMP-2 has higher levels of bioactivity than soluble rhBMP-2. Based on this finding, we suggest that materials or devices (keratin, collagen, or otherwise) that release rhBMP-2 should emphasize the matrix-associated component and use only enough soluble rhBMP-2 to initiate a cellular response in

which mesenchymal stem cells or osteoprogenitor cells can be recruited to the defect site (location of the material scaffold). Such an approach could reduce the formation of ectopic bone, and is consistent with a previous result observed by our group [13]. An interpretation from the results can be that the osteoinductive role of rhBMP-2 can be controlled when the degree of cross-linking is decreased thereby effecting its degradation and providing a more extracellular matrix framework. Also, it has been reported that to enhance the efficacy of bone regeneration for treatment of larger bone defects, sustained delivery of rhBMP-2 [14], [15] is required which can be achieved using the keratin material. The sustained delivery would make rhBMP-2 available at later time points which would help in recruiting additional osteoprogenitor cells at the injury site thus enhancing bone regeneration [16]. Again, the findings of this thesis work seem consistent with those previously suggested.

III.2. Future work

This thesis work has provided some insights about the use of the keratin biomaterials as potential next-generation carrier of rhBMP-2. In particular, these studies should shed some light on the effects that can be expected through control over the degradation rate, release of rhBMP-2 and rhBMP-2 bioactivity. Clearly, all questions were not answered about the use of keratin as a carrier for the delivery of rhBMP-2 and there are several areas where future studies can be performed. The main objective of developing any carrier system for the delivery of growth factor is the capability to retain the growth factor at the injury site such that the growth factor can exhibit its desired biological effect [17]. From our results it appears that KOS has higher total bioactivity than KTN (combining bioactivities associated with the soluble and matrix-associated rhBMP-2). However, higher bioactivity levels may not be totally desirable given the supraphysiological dose of rhBMP-2 used and previous observations (as noted above) of side effects such as ectopic bone growth. One advantage to the keratin mixtures is that a mixture of, for example, 50:50 KOS:KTN is that interplay of bioactivity between the soluble and matrix-associated forms could be optimized to achieve appropriate healing while minimizing undesirable side effects. That is, this would allow similar levels of activity to be achieved with a lower dose, reducing the risk of side effects/complications like ectopic bone formation.

Many factors must be taken into consideration in the development of keratin biomaterials or other materials as a potential next generation carrier for delivery of rhBMP-2. One

consideration is the chemical composition of the carrier in that it should not have toxic effects. Moreover, these materials should show minimal immune response which could be generated when in contact with the host. Indeed, these aspects are likely responsible for the use of natural polymer carriers in the clinic, and previous studies with keratin indicate low levels of toxicity [21].

The physical characteristic of the biomaterial are important, as well. For example, the material must have sufficient porosity to allow cellular infiltration and remodeling of the scaffold matrix. It must also have suitable mechanical properties to allow it to be implanted and retained at the implant site. Again, as with collagen, keratin materials can achieve these effects.

Ultimately, however, any carrier should ideally lead to bone regeneration that leads to bone architecture similar to native (uninjured/non-diseased) bone. In this work, we have quantitatively analyzed the production of alkaline phosphatase enzyme which is a marker indicating the mineralization process. However, this is only one marker for assessing the bioactivity. Other markers like Core binding factor a-1 (Cbfa1 or Runx2) which indicates the early stage of osteoblast differentiation and Osteopontin (OPN) which shows the differentiation of osteoblast thus pointing the early osteoprogenitor cell marker can be analyzed to provide more information on the effects at earlier or later timepoints in the cellular response to the rhBMP-2 delivered from materials. Also, the MC3T3-E1 cells could be exposed to rhBMP-2 for a longer period of time and as a result osteocalcin secretion and mineralization composition can be determined. It should be noted, as well, that we used MC3T3-E1 pre-osteoblasts, but these cells may have important differences from the cells that respond to a defect and subsequent implanted materials/rhBMP-2 such as osteprogenitor cells or mesenchymal stem cells. As such, studies with other cells and their response to these materials and the delivered rhBMP-2 would be appropriate.

In our pilot *in vivo* study, we noted that the lower limit of detection was not suitable for our purposes. However, we used micro-CT scans as an outcome measure. It should be possible to use lower doses of rhBMP-2 to assess bioactivity, but using (for example) histological evaluation rather than micro-CT as an outcome measure. That said, in order to achieve histologically identifiable effects, longer time courses (e.g., 8 weeks) may be required, which would lead to degradation of the carrier and thus a challenge in determining whether soluble (released) or matrix-associated rhBMP-2 is responsible for the observed effect. As such, we

suggest that the *in vitro* alkaline phosphatase assay is a good starting point and that *in vitro* assays may be better suited to answer this question due to the shorter timeframe over which observations can be made.

The approaches described here could also be used to investigate other methods by which disulfide crosslinking is modified, such as another recent study by our group in which disulfide crosslinking was modified by alkylation of cysteine residues to give so-called modified keratins (MKTN). Future research can also investigate the effects of bioactivity when rhBMP-2 is released from MKTN. Given the increased hydrophobicity associated with the alkylation process, it is possible that this modification may further enhance the bioactivity of both the soluble and matrix-associated rhBMP-2. So, the immediate studies following this research can include an assay that will quantify the amount of rhBMP-2 that has been degraded/denatured. This may lead to further modifications to keratin (or other material) carriers to better preserve rhBMP-2 bioactivity. One approach to this is the use of heparan sulfate proteoglycans [18] as a means to preserve activity needed for differentiation, and proliferation of osteoblast cells into fully mature osteocytes.

The main objective to study keratin as a potential carrier for delivery of rhBMP- 2 is to reduce the rapid burst of BMP-2 as well as to ensure its sustained delivery over time. Our results indicate that the matrix-bound rhBMP-2 is responsible for bone formation, so by altering the percentage of disulfide cross link and decreasing the amount of BMP-2 used, the rapid burst of rhBMP-2 and subsequent ectopic bone formation might be reduced, thus enhancing efficacy of rhBMP-2. Approaches that achieve similar levels of bone regeneration with lower rhBMP-2 (achieved by methods mentioned above or as yet unknown means) may allow reduction of ectopic bone growth. This, in turn, may allow for the use of synthetic bone grafts in a wider spectrum of uses beyond those for which it is currently approved. Finally, this would lead to the potential for improved patient care through the use of an off-the-shelf system that does not require autograft or allograft sourcing of materials to promote bone regeneration.

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